1274571

THE UNITED STATES OF AMERICA

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January 19, 2005

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APPLICATION NUMBER: *60/526,664*
FILING DATE: *December 03, 2003*
RELATED PCT APPLICATION NUMBER: *PCT/US04/40234*

Certified by

Jon W. Dudas

Under Secretary of Commerce
for Intellectual Property
and Director of the United States
Patent and Trademark Office

22386 U.S.PTO 60/526664

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EL983812154US

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Philip J.	Fay	43 Split Rock Road Pittsford, New York 14534
Hironao	Wakabayashi	136 Greystone Lane #3 Rochester, New York 14618

☐ *Additional inventors are being named on the ____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

RECOMBINANT FACTOR VIII HAVING INCREASED SPECIFIC ACTIVITY

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

☐ Customer Number [ ] → *Place Customer Number Bar Code Label here*

OR *Type Customer Number here*

☒ Firm *or* Individual Name	Nixon Peabody LLP				
Address	Clinton Square, P.O. Box 31051				
City	Rochester	State	NY	ZIP	14603-1051
Country	USA	Telephone	(585) 263-1128	Fax	(585) 263-1600

ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 65 ☐ CD(s), Number [ ]

☐ Drawing(s) *Number of Sheets* [ ] ☐ Other (specify) [ ]

☐ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ Applicant claims small entity status. See 37 CFR 1.27.

☒ A check or money order is enclosed to cover the filing fees

☒ The Commissioner is hereby authorized to charge fees which may be required, or credit any overpayment to Deposit Account Number: 14-1138

☐ Payment by credit card. Form PTO-2038 is attached.

FILING FEE AMOUNT ($): $80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.

☒ Yes, the name of the U.S. Government agency and the Government contract number are: NIH Grant HL 38199 and NIH Grant HL 30616.

Respectfully submitted, Date 12/3/03

SIGNATURE _____ REGISTRATION NO. *(if appropriate)* 40,087

TYPED or PRINTED NAME Edwin V. Merkel Docket Number: 176/61700 (1265)

TELEPHONE (585) 263-1128

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

FEE TRANSMITTAL FOR FY 2003

Patent fees are subject to annual revision.

☒ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 80

Complete if Known	
Application Number	To be Assigned
Filing Date	Herewith
First Named Inventor	Fay et al.
Examiner Name	
Art Unit	
Attorney Docket No.	176/61700 (1265)

METHOD OF PAYMENT *(check all that apply)*

☒ Check ☐ Credit Card ☐ Money Order ☐ Other ☐ None

☐ Deposit Account:

Deposit Account Number: 14-1138

Deposit Account Name: Nixon Peabody LLP

The Commissioner is authorized to: *(check all that apply)*

☐ Charge fee(s) indicated below ☒ Credit any overpayments

☒ Charge any additional fee(s)

☐ Charge fee(s) indicated below, **except for the filing fee** to the above-identified deposit account.

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	770	2001	385	Utility filing fee	
1002	340	2002	170	Design filing fee	
1003	530	2003	265	Plant filing fee	
1004	770	2004	385	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	80

SUBTOTAL (1) ($) 80

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

		Extra Claims		Fee from below		Fee Paid
Total Claims	-20** =		X		=	0
Independent Claims	-3** =		X		=	0
Multiple Dependent			X		=	0

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	86	2201	43	Independent claims in excess of 3
1203	290	2203	145	Multiple dependent claim, if not paid
1204	86	2204	43	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 0

**or number previously paid, if greater; For Reissues, see above

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	
1051	130	2051	65	Surcharge – late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	420	2252	210	Extension for reply within second month	
1253	950	2253	475	Extension for reply within third month	
1254	1,480	2254	740	Extension for reply within fourth month	
1255	2,010	2255	1,005	Extension for reply within fifth month	
1401	330	2401	165	Notice of Appeal	
1402	330	2402	165	Filing a brief in support of an appeal	
1403	290	2403	145	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive – unavoidable	
1453	1,330	2453	665	Petition to revive – unintentional	
1501	1,330	2501	665	Utility issue fee (or reissue)	
1502	480	2502	240	Design issue fee	
1503	640	2503	320	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	770	2809	385	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	770	2810	385	For each additional invention to be examined (37 CFR 1.129(b))	
1801	770	2801	385	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) _____

*Reduced by Basic Filing Fee Paid

SUBTOTAL (3) ($) 0

CERTIFICATE OF MAILING OR TRANSMISSION [37 CFR 1.8(a)]

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Name *(Print/Type)*	Edwin V. Merkel	Registration No. *(Attorney/Agent)*	40,087	Telephone	(585) 263-1128
Signature				Date	12/3/03

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R732269.1

EXPRESS MAIL CERTIFICATE

DOCKET NO.: **176/61700 (1265)**

APPLICANTS: **Philip J. Fay and Hironao Wakabayashi**

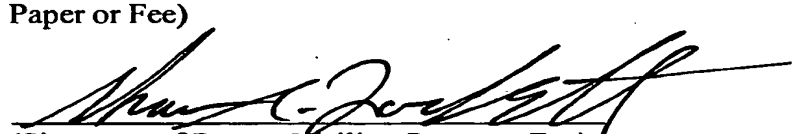
TITLE: **RECOMBINANT FACTOR VIII HAVING INCREASED SPECIFIC ACTIVITY**

Certificate is attached to the **Provisional Patent Application (65 pages)** of the above-identified application.

"EXPRESS MAIL" NUMBER: **EL983812154US**
DATE OF DEPOSIT: **December 3, 2003**

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to Mail Stop Provisional Patent Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Shawn A. Lockett
(Typed or Printed Name of Person Mailing Paper or Fee)



(Signature of Person Mailing Paper or Fee)

Title: **RECOMBINANT FACTOR VIII HAVING INCREASED SPECIFIC ACTIVITY**

Inventors: **PHILIP J. FAY**
HIRONAO WAKABAYASHI

Docket No.: **176/61700 (1265)**

PROVISIONAL APPLICATION

RECOMBINANT FACTOR VIII HAVING INCREASED SPECIFIC ACTIVITY

The present invention was made with funding received from the National Institutes of Health under grants HL 38199 and HL 30616. The U.S. government may retain certain rights in this invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a recombinant factor VIII having increased specific (or pro-coagulant) activity as compared to wild-type factor VIII. The recombinant factor VIII includes a point mutation in or near at least one calcium binding site of a wild-type factor VIII. As used herein, "in or near" means within about five amino acid residues from a residue that directly interacts with Ca^{2+} or Mn^{2+} ions.

The recombinant factor VIII of the present invention can be prepared by modifying the amino acid sequence of a wild-type factor VIII or a mutant factor VIII that has otherwise been modified to affect other properties of the factor VIII, such as antigenicity, circulating half-life, protein secretion, affinity for factor IXa and/or factor X, altered factor VIII-inactivation cleavage sites, stability of the activated factor VIII form, immunogenicity, shelf-life, etc.

Suitable wild-type factor VIII that can be modified in accordance with the present invention can be from various animals including, without limitation, mammals such as humans, rats, mice, guinea pigs, dogs, cats, monkeys, chimpanzees, orangutans, cows, horses, sheep, pigs, goats, rabbits, and chickens.

By way of example, the human factor VIII cDNA nucleotide and predicted amino acid sequences are shown below in SEQ ID NOs: 1 and 2, respectively. Human factor VIII is synthesized as an approximately 300 kDa single chain protein with internal sequence homology that defines the "domain" sequence NH_2-A1-A2-B-A3-C1-C2-COOH. In a factor VIII molecule, a "domain," as used herein, is a continuous sequence of amino acids that is defined by internal amino acid sequence identity and sites of proteolytic cleavage by thrombin. Unless otherwise specified, factor VIII domains include the following amino acid residues, when the sequences are aligned with the human amino acid sequence (SEQ ID NO: 2):

A1, residues Ala_1-Arg_{372};

A2, residues Ser_{373}-Arg_{740};

B, residues Ser_{741}-Arg_{1648};

A3, residues Ser_{1690}-Ile_{2032};

C1, residues Arg_{2033}-Asn_{2172};

C2, residues Ser_{2173}-Tyr_{2332}.

The A3-C1-C2 sequence includes residues Ser_{1690}-Tyr_{2332}. The remaining sequence, residues Glu_{1649}-Arg_{1689}, is usually referred to as the factor VIII light chain activation peptide. Factor VIII is proteolytically activated by thrombin or factor Xa, which dissociates it from von Willebrand factor, forming factor VIIIa, which has procoagulant function. The biological function of factor VIIIa is to increase the catalytic efficiency of factor IXa toward factor X activation by several orders of magnitude. Thrombin-activated factor VIIIa is a 160 kDa A1/A2/A3-C1-C2 heterotrimer that forms a complex with factor IXa and factor X on the surface of platelets or monocytes. A "partial domain" as used herein is a continuous sequence of amino acids forming part of a domain.

SEQ ID NO: 1 (encoding wild-type human factor VIII):

gccaccagaagatactacctgggtgcagtggaactgtcatgggactatatgcaaagtgatctcggtgagct
gcctgtggacgcaagatttcctcctagagtgccaaaatcttttccattcaacacctcagtcgtgtacaaaa
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ggatggaggttctgggctgcgaggcacaggacctctactga

SEQ ID NO: 2 (wild-type human factor VIII):

ATRRYYLGAVELSWDYMQSDLGELPVDARFPPRVPKSFPFNTSVVYKKTLFVEFTVHLFNIAKPRPPWMGL
LGPTIQAEVYDTVVITLKNMASHPVSLHAVGVSYWKASEGAEYDDQTSQREKEDDKVFPGGSHTYVWQVLK
ENGPMASDPLCLTYSYLSHVDLVKDLNSGLIGALLVCREGSLAKEKTQTLHKFILLFAVFDEGKSWHSETK
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NVVLPQIHTVTGTKNFMKNLFLLSTRQNVEGSYEGAYAPVLQDFRSLNDSTNRTKKHTAHFSKKGEEENLE
GLGNQTKQIVEKYACTTRISPNTSQQNFVTQRSKRALKQFRLPLEETELEKRIIVDDTSTQWSKNMKHLTP
STLTQIDYNEKEKGAITQSPLSDCLTRSHSIPQANRSPLPIAKVSSFPSIRPIYLTRVLFQDNSSHLPAAS
YRKKDSGVQESSHFLQGAKKNNLSLAILTLEMTGDQREVGSLGTSATNSVTYKKVENTVLPKPDLPKTSGK
VELLPKVHIYQKDLFPTETSNGSPGHLDLVEGSLLQGTEGAIKWNEANRPGKVPFLRVATESSAKTPSKLL
DPLAWDNHYGTQIPKEEWKSQEKSPEKTAFKKKDTILSLNACESNHAIAAINEGQNKPEIEVTWAKQGRTE
RLCSQNPPVLKRHQREITRTTLQSDQEEIDYDDTISVEMKKEDFDIYDEDENQSPRSFQKKTRHYFIAAVE

RLWDYGMSSSPHVLRNRAQSGSVPQFKKVVFQEFTDGSFTQPLYRGELNEHLGLLGPYIRAEVEDNIMVTF
RNQASRPYSFYSSLISYEEDQRQGAEPRKNFVKPNETKTYFWKVQHHMAPTKDEFDCKAWAYFSDVDLEKD
VHSGLIGPLLVCHTNTLNPAHGRQVTVQEFALFFTIFDETKSWYFTENMERNCRAPCNIQMEDPTFKENYR
FHAINGYIMDTLPGLVMAQDQRIRWYLLSMGSNENIHSIHFSGHVFTVRKKEEYKMALYNLYPGVFETVEM
LPSKAGIWRVECLIGEHLHAGMSTLFLVYSNKCQTPLGMASGHIRDFQITASGQYGQWAPKLARLHYSGSI
NAWSTKEPFSWIKVDLLAPMIIHGIKTQGARQKFSSLYISQFIIMYSLDGKKWQTYRGNSTGTLMVFFGNV
DSSGIKHNIFNPPIIARYIRLHPTHYSIRSTLRMELMGCDLNSCSMPLGMESKAISDAQITASSYFTNMFA
TWSPSKARLHLQGRSNAWRPQVNNPKEWLQVDFQKTMKVTGVTTQGVKSLLTSMYVKEFLISSSQDGHQWT
LFFQNGKVKVFQGNQDSFTPVVNSLDPPLLTRYLRIHPQSWVHQIALRMEVLGCEAQDLY

Suitable calcium binding sites that are available for mutation in accordance with the present invention can be located within any one of the A1, A2, A3, C1, and/or C2 domains of the activated wild-type factor VIII. In a preferred embodiment, the calcium binding site is located in the A1 domain, particularly between residues 110-126 as identified (underlined) in SEQ ID NO: 2 above.

Exemplary recombinant factor VIII includes a point mutation involving a substitution of the glutamic acid residue at position 113 of SEQ ID NO: 2 (shown in bold typeface in SEQ ID NO: 2), with another residue that is other than aspartic acid. In particular, the substitutions at position 113 of SEQ ID NO: 2 can include, without limitation, the following substitutions: E113A, E113V, E113I, E113N, E113L, E113G, and E113M. Of these, the E113A substitution is preferred, having a specific activity that is at least about twice as great as wild-type factor VIII. The substitution at the E113 residue can also be made using the various modified forms and/or derivatives of the substituting amino acid residues noted above (see, e.g., *Chem Files,* Vol. 2, No. 4, "Unnatural Amino Acids II: The latest Update on New Tools for Drug Discovery" (available from Sigma-Aldrich), which is hereby incorporated by reference in its entirety).

Another property of the recombinant factor VIII of the present invention is its higher binding affinity for Ca^{2+}, Mn^{2+}, or possibly other cations as compared to that of the wild-type factor VIII.

Suitable mutant factor VIII sequences that can be modified in accordance with the present invention can also include any previously known or subsequently

identified mutant factor VIII sequences that have modified properties with regard to various attributes, including, without limitation, antigenicity, circulating half-life, protein secretion, affinity for factor IXa and/or factor X, altered factor VIII-inactivation cleavage sites, stability of the activated factor VIII form, immunogenicity, and shelf-life.

One example of a suitable mutant factor VIII that can be modified in accordance with the present invention is a B-domainless factor VIII that contains aa 1-740 and 1690-2332 of SEQ ID NO: 2. (see, e.g., U.S. Patent No. 6,458,563 to Lollar, which is hereby incorporated by reference in its entirety). Preferably, the recombinant B-domainless factor VIII contains one of the substitutions at position 113 identified herein.

A second example of a suitable mutant factor VIII that can be modified in accordance with the present invention is a chimeric human/porcine factor VIII that contains one or more porcine amino acid residues as substitution(s) for human amino acid residues that are responsible for the antigenicity of human factor VIII. In particular, porcine residue substitutions can include, without limitation, one or more of the following: R484A, P485A, Y487A, P488A, R489A, P492A, V495A, F501A, and/or I508A (U.S. Patent No. 5,859,204 to Lollar, which is hereby incorporated by reference in its entirety). Preferably, the recombinant chimeric human/porcine factor VIII contains one of the substitutions at position 113 identified herein.

A third example of a suitable mutant factor VIII that can be modified in accordance with the present invention is a factor VIII that is characterized by greater stability of activated factor VIII by virtue of fused A2 and A3 domains. In particular, a factor VIII can be modified by substituting cysteine residues at positions 664 and 1826, resulting in a mutant factor VIII that includes a Cys664-Cys1826 disulfide bond that covalently links the A2 and A3 domains (Gale et al., "An Engineered Interdomain Disulfide Bond Stabilizes Human Blood Coagulation Factor VIIIa," *J. Thrombosis and Haemostasis* 1(9):1966-1971 (2003), which is hereby incorporated by reference in its entirety). Preferably, the recombinant fused domain (A2-A3) factor VIII contains one of the substitutions at position 113 identified herein.

A fourth example of a suitable mutant factor VIII that can be modified in accordance with the present invention is a factor VIII with altered inactivation cleavage

sites (see, e.g., Amano et al., "Mutation at Either Arg336 or Arg562 in Factor VIII is Insufficient for Complete Resistance to Activated Protein C (APC)-Mediated Inactivation: Implications for the APC Resistance Test," *Thrombosis & Haemostasis* 79(3):557-63 (1998), which is hereby incorporated by reference in its entirety). These alterations can be used to decrease the mutant factor VIII's susceptibility to cleavage enzymes that normally inactivate the wild type factor VIII.

A fifth example of a suitable mutant factor VIII that can be modified in accordance with the present invention is a factor VIII that has enhanced affinity for factor IXa (see, e.g., Fay et al., "Factor VIIIa A2 Subunit Residues 558-565 Represent a Factor IXa Interactive Site," *J. Biol. Chem.* 269(32):20522-7 (1994); Bajaj et al., "Factor IXa: Factor VIIIa Interaction. Helix 330-338 of Factor IXa Interacts with Residues 558-565 and Spatially Adjacent Regions of the A2 Subunit of Factor VIIIa," *J. Biol. Chem.* 276(19):16302-9 (2001); and Lenting et al., "The Sequence Glu1811-Lys1818 of Human Blood Coagulation Factor VIII Comprises a Binding Site for Activated Factor IX," *J. Biol. Chem.* 271(4):1935-40 (1996), which are hereby incorporated by reference in their entirety) and/or factor X (see, e.g., Lapan et al., "Localization of a Factor X Interactive Site in the A1 Subunit of Factor VIIIa," *J. Biol. Chem.* 272:2082-88 (1997), which is hereby incorporated by reference in its entirety).

A sixth example of a suitable mutant factor VIII that can be modified in accordance with the present invention is a factor VIII that is modified to enhance secretion of the factor VIII (see, e.g., Swaroop et al., "Mutagenesis of a Potential Immunoglobulin-Binding Protein-Binding Site Enhances Secretion of Coagulation Factor VIII," *J. Biol. Chem.* 272(39):24121-4 (1997), which is hereby incorporated by reference in its entirety).

A seventh example of a suitable mutant factor VIII that can be modified in accordance with the present invention is a factor VIII with an increased circulating half-life. This modification can be made using various approaches, including, without limitation, by reducing interactions with heparan sulfate (Sarafanov et al., "Cell Surface Heparan Sulfate Proteoglycans Participate in Factor VIII Catabolism Mediated by Low Density Lipoprotein Receptor-Related Protein," *J. Biol. Chem.* 276(15):11970-9

(2001),which is hereby incorporated by reference in its entirety) and/or low-density lipoprotein receptor-related protein ("LRP") (Saenko et al., "Role of the Low Density Lipoprotein-Related Protein Receptor in Mediation of Factor VIII Catabolism," *J. Biol. Chem.* 274(53):37685-92 (1999); and Lenting et al., "The Light Chain of Factor VIII Comprises a Binding Site for Low Density Lipoprotein Receptor-Related Protein," *J. Biol. Chem.* 274(34):23734-9 (1999), which are hereby incorporated by reference in their entirety)

Further, the mutant factor VIII can be modified to take advantage of various advancements regarding recombinant coagulation factors generally (see, e.g., Saenko et al., "The Future of Recombinant Coagulation Factors," *J. Thrombosis and Haemostasis* 1:922-930 (2003), which is hereby incorporated by reference in its entirety).

The recombinant factor VIII of the present invention can be modified at position 113, as well as be modified to be B-domainless, to be chimeric, to have fused A2-A3 domains, to have altered inactivation cleavage sites, to have enhanced factor IXa and/or factor X affinity, to have enhanced secretion, to have an increased circulating half-life, or to possess any two of such modifications in addition to the modification at position 113.

The recombinant factor VIII is preferably produced in a substantially pure form. In a particular embodiment, the substantially pure recombinant factor VIII is at least about 80% pure, more preferably at least 90% pure, most preferably at least 95% pure. A substantially pure recombinant factor VIII can be obtained by conventional techniques well known in the art. Typically, the substantially pure recombinant factor VIII is secreted into the growth medium of recombinant host cells. Alternatively, the substantially pure recombinant factor VIII is produced but not secreted into growth medium. In such cases, to isolate the substantially pure recombinant factor VIII, the host cell carrying a recombinant plasmid is propagated, lysed by sonication, heat, or chemical treatment, and the homogenate is centrifuged to remove cell debris. The supernatant is then subjected to sequential ammonium sulfate precipitation. The fraction containing the substantially pure recombinant factor VIII is subjected to gel filtration in an appropriately sized dextran or polyacrylamide column to separate the recombinant factor VIII. If

necessary, a protein fraction (containing the substantially pure recombinant factor VIII) may be further purified by high performance liquid chromatography ("HPLC").

Another aspect of the present invention relates to an isolated nucleic acid molecule that encodes the recombinant factor VIII of the present invention. The isolated nucleic acid molecule encoding the recombinant factor VIII can be either RNA or DNA.

In one embodiment, the isolated nucleic acid molecule can have a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 2 as modified with one of the substitutions at position 113 (i.e., possessing one to three nucleotide substitutions within codon 113 of SEQ ID NO: 1 (nt 337-339).

In another embodiment, the isolated nucleic acid molecule can have a nucleotide sequence encoding a B-domainless factor VIII of the type described above, as modified with one of the substitutions at position 113.

In another embodiment, the isolated nucleic acid molecule can have a nucleotide sequence encoding a chimeric human/porcine of the type described above, as modified with one of the substitutions at position 113.

In a further embodiment, the isolated nucleic acid molecule can have a nucleotide sequence encoding a fused A2-A3 domain factor VIII of the type described above, as modified with one of the substitutions at position 113.

In another embodiment, the isolated nucleic acid molecule can have a nucleotide sequence encoding a factor VIII whose inactivation sites have been modified as described above, as further modified with one of the substitutions at position 113.

In yet another embodiment, the isolated nucleic acid molecule can have a nucleotide sequence encoding a factor VIII whose affinity for factor IXa and/or factor X has been enhanced, as further modified with one of the substitutions at position 113.

In a still further embodiment, the isolated nucleic acid molecule can have a nucleotide sequence encoding a factor VIII whose affinity for various serum-binding proteins has been altered to increase its circulating half-life, as further modified with one of the substitutions at position 113.

In a further embodiment, the isolated nucleic acid molecule can have a nucleotide sequence encoding a factor VIII that has increased secretion in culture, as further modified with one of the substitutions at position 113.

In yet another embodiment, the isolated nucleic acid molecule encodes a recombinant factor VIII that is modified at position 113 and is also modified to possess any two or more of the following: modified to be B-domainless, modified to be chimeric, modified to have fused A2-A3 domains, modified to have altered inactivation cleavage sites, modified to have enhanced factor IXa and/or factor X affinity, modified to have enhanced secretion, and modified to have an increased circulating half-life.

Another aspect of the present invention relates to a recombinant DNA expression system that includes an isolated DNA molecule of the present invention, which expression system encodes a recombinant factor VIII. In one embodiment, the DNA molecule is in sense orientation relative to a promoter.

A further aspect of the present invention relates to a host cell including an isolated nucleic acid molecule encoding the recombinant factor VIII of the present invention. In a particular embodiment, the host cell can contain the isolated nucleic acid molecule in DNA molecule form, either as a stable plasmid or as a stable insertion or integration into the host cell genome. In another embodiment, the host cell can contain a DNA molecule in an expression system. Suitable host cells can be, without limitation, animal cells (e.g., baby hamster kidney ("BHK") cells), bacterial cells (e.g., *E. coli*), insect cells (e.g., Sf9 cells), fungal cells, yeast cells (e.g., *Saccharomyces* or *Schizosaccharomyces*), plant cells (e.g., *Arabidopsis* or tobacco cells), or algal cells.

The recombinant DNA expression system and host cells can be produced using various recombinant techniques well-known in the art, as further discussed below.

The DNA molecule encoding the recombinant factor VIII of the present invention can be incorporated in cells using conventional recombinant DNA technology. Generally, this involves inserting the DNA molecule into an expression system to which the DNA molecule is heterologous (i.e., not normally present). The heterologous DNA molecule is inserted into the expression system or vector in sense orientation and correct reading frame. The vector contains the necessary elements for the transcription and

translation of the inserted protein-coding sequences. Thus, one embodiment of the present invention provides a DNA construct containing the isolated nucleic acid of the present invention, which is operably linked to both a 5' promoter and a 3' regulatory region (i.e., transcription terminator) capable of affording transcription and expression of the encoded recombinant factor VIII of the present invention in host cells or host organisms.

With respect to the recombinant expression system of the present invention, an expression vector containing a DNA molecule encoding the recombinant factor VIII of the present invention can be made using common techniques in the art. The nucleic acid molecules of the present invention can be inserted into any of the many available expression vectors using reagents that are well known in the art. In preparing a DNA vector for expression, the various DNA sequences may normally be inserted or substituted into a bacterial plasmid. Any convenient plasmid may be employed, which will be characterized by having a bacterial replication system, a marker which allows for selection in a bacterium, and generally one or more unique, conveniently located restriction sites. The selection of a vector will depend on the preferred transformation technique and target host for transformation.

A variety of host-vector systems may be utilized to express the recombinant factor VIII-encoding sequence(s). Primarily, the vector system must be compatible with the host cell used. Host-vector systems include but are not limited to the following: bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, adeno-associated virus, etc.); insect cell systems infected with virus (e.g., baculovirus); and plant cells infected by bacteria (~~e.g.~~, *Agrobacterium*). The expression elements of these vectors vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

When recombinantly produced, the factor VIII protein or polypeptide (or fragment or variant thereof) is expressed in a recombinant host cell, typically, although not exclusively, a eukaryote.

Suitable vectors for practicing the present invention include, but are not limited to, the following viral vectors such as lambda vector system gt11, gtWES.tB, Charon 4, and plasmid vectors such as pCMV, pBR322, pBR325, pACYC177, pACYC184, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II SK +/- or KS +/- (see "Stratagene Cloning Systems" Catalog (1993)), pQE, pIH821, pGEX, pET series (Studier et al, "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes," *Methods in Enzymology* 185:60-89 (1990), which is hereby incorporated by reference in its entirety), and any derivatives thereof. Any appropriate vectors now known or later described for genetic transformation are suitable for use with the present invention.

Recombinant molecules can be introduced into cells via transformation, particularly transduction, conjugation, mobilization, or electroporation. The DNA sequences are cloned into the vector using standard cloning procedures in the art, as described by Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor, N.Y.: Cold Springs Laboratory, (1982), which is hereby incorporated by reference in its entirety.

U.S. Patent No. 4,237,224 issued to Cohen and Boyer, which is hereby incorporated by reference in its entirety, describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including prokaryotic organisms and eukaryotic cells grown in tissue culture.

Different genetic signals and processing events control many levels of gene expression (e.g., DNA transcription and messenger RNA (mRNA) translation).

Transcription of DNA is dependent upon the presence of a promoter which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes mRNA synthesis. The DNA sequences of eukaryotic promoters differ from those of prokaryotic promoters. Furthermore, eukaryotic promoters and accompanying genetic signals may not be recognized in or may not function in a prokaryotic system,

and, further, prokaryotic promoters are not recognized and do not function in eukaryotic cells.

Similarly, translation of mRNA in prokaryotes depends upon the presence of the proper prokaryotic signals which differ from those of eukaryotes. Efficient translation of mRNA in prokaryotes requires a ribosome binding site called the Shine-Dalgarno ("SD") sequence on the mRNA. This sequence is a short nucleotide sequence of mRNA that is located before the start codon, usually AUG, which encodes the amino-terminal methionine of the protein. The SD sequences are complementary to the 3'-end of the 16S rRNA (ribosomal RNA) and probably promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct positioning of the ribosome. For a review on maximizing gene expression, see Roberts and Lauer, *Methods in Enzymology* 68:473 (1979), which is hereby incorporated by reference in its entirety.

Promoters vary in their "strength" (i.e., their ability to promote transcription). For the purposes of expressing a cloned gene, it is generally desirable to use strong promoters in order to obtain a high level of transcription and, hence, expression of the gene. Depending upon the host cell system utilized, any one of a number of suitable promoters may be used. For instance, when cloning in *Escherichia coli*, its bacteriophages, or plasmids, promoters such as the T7 phage promoter, *lac* promoter, *trp* promoter, *rec*A promoter, ribosomal RNA promoter, the P_R and P_L promoters of coliphage lambda and others, including but not limited, to *lac*UV5, *omp*F, *bla*, *lpp*, and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a hybrid *trp-lac*UV5 (*tac*) promoter or other *E. coli* promoters produced by recombinant DNA or other synthetic DNA techniques may be used to provide for transcription of the inserted gene.

Bacterial host cell strains and expression vectors may be chosen which inhibit the action of the promoter unless specifically induced. In certain operations, the addition of specific inducers is necessary for efficient transcription of the inserted DNA. For example, the *lac* operon is induced by the addition of lactose or IPTG (isopropylthio-beta-D-galactoside). A variety of other operons, such as *trp*, *pro*, etc., are under different controls.

Specific initiation signals are also required for efficient gene transcription and translation in prokaryotic cells. These transcription and translation initiation signals may vary in "strength" as measured by the quantity of gene specific messenger RNA and protein synthesized, respectively. The DNA expression vector, which contains a promoter, may also contain any combination of various "strong" transcription and/or translation initiation signals. For instance, efficient translation in *E. coli* requires an SD sequence about 7-9 bases 5' to the initiation codon ("ATG") to provide a ribosome binding site. Thus, any SD-ATG combination that can be utilized by host cell ribosomes may be employed. Such combinations include but are not limited to the SD-ATG combination from the *cro* gene or the *N* gene of coliphage lambda, or from the *E. coli* tryptophan E, D, C, B or A genes. Additionally, any SD-ATG combination produced by recombinant DNA or other techniques involving incorporation of synthetic nucleotides may be used.

In one embodiment, the nucleic acid molecule of the present invention is incorporated into an appropriate vector in the sense direction, such that the open reading frame is properly oriented for the expression of the encoded protein under control of a promoter of choice. This involves the inclusion of the appropriate regulatory elements into the DNA-vector construct. These include non-translated regions of the vector, useful promoters, and 5' and 3' untranslated regions which interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used.

A constitutive promoter is a promoter that directs expression of a gene throughout the development and life of an organism.

An inducible promoter is a promoter that is capable of directly or indirectly activating transcription of one or more DNA sequences or genes in response to an inducer. In the absence of an inducer, the DNA sequences or genes will not be transcribed.

The DNA construct of the present invention can also includes an operable 3' regulatory region, selected from among those which are capable of providing correct transcription termination and polyadenylation of mRNA for expression in the host cell of choice, operably linked to a DNA molecule which encodes for a protein of choice.

The vector of choice, promoter, and an appropriate 3' regulatory region can be ligated together to produce the DNA construct of the present invention using well known molecular cloning techniques as described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Press, NY (1989), and Ausubel, F. M. et al. *Current Protocols in Molecular Biology*, New York, N.Y: John Wiley & Sons (1989), which are hereby incorporated by reference in their entirety.

As noted, one alternative to the use of prokaryotic host cells is the use of eukaryotic host cells, such as mammalian cells, which can also be used to recombinantly produce the recombinant factor VIII of the present invention. Mammalian cells suitable for carrying out the present invention include, among others: COS (e.g., ATCC No. CRL 1650 or 1651), BHK (e.g., ATCC No. CRL 6281), CHO (ATCC No. CCL 61), HeLa (e.g., ATCC No. CCL 2), 293 (ATCC No. 1573), CHOP, and NS-1 cells.

Suitable expression vectors for directing expression in mammalian cells generally include a promoter, as well as other transcription and translation control sequences known in the art. Common promoters include SV40, MMTV, metallothionein-1, adenovirus Ela, CMV, immediate early, immunoglobulin heavy chain promoter and enhancer, and RSV-LTR.

Once the DNA construct of the present invention has been prepared, it is ready to be incorporated into a host cell. Accordingly, another aspect of the present invention relates to a method of making a recombinant cell. Basically, this method is carried out by transforming a host cell with a DNA construct of the present invention under conditions effective to yield transcription of the DNA molecule in the host cell. Recombinant molecules can be introduced into cells via transformation, particularly transduction, conjugation, mobilization, or electroporation.

In view of the recombinant technology discussed herein, another aspect of the present invention relates to a method of making a recombinant factor VIII of the

present invention. This method involves growing a host cell of the present invention under conditions whereby the host cell expresses the recombinant factor VIII. The recombinant factor VIII is then isolated. In one embodiment, the host cell is grown *in vitro* in a growth medium. In a particular embodiment, suitable growth media can include, without limitation, a growth medium containing a von Willebrand Factor (referred to herein as "VWF"). In this embodiment, the host cell can contain a transgene encoding a VWF or the VWF can be introduced to the growth medium as a supplement. VWF in the growth medium will allow for greater expression levels of the recombinant factor VIII. Once the recombinant factor VIII is secreted into the growth medium, it can then be isolated from the growth medium using techniques well-known by those of ordinary skill in the relevant recombinant DNA and protein arts (including those described herein). In another embodiment, the method of making the recombinant factor VIII of the present invention further involves disrupting the host cell prior to isolation of the recombinant factor VIII. In this embodiment, the recombinant factor VIII is isolated from cellular debris.

When an expression vector is used for purposes of *in vivo* transformation to induce factor VIII expression in a target cell, promoters of varying strength can be employed depending on the degree of enhancement desired. One of skill in the art can readily select appropriate mammalian promoters based on their strength as a promoter. Alternatively, an inducible promoter can be employed for purposes of controlling when expression or suppression of factor VIII is desired. One of skill in the art can readily select appropriate inducible mammalian promoters from those known in the art. Finally, tissue specific mammalian promoters can be selected to restrict the efficacy of any gene transformation system to a particular tissue. Tissue specific promoters are known in the art and can be selected based upon the tissue or cell type to be treated.

Another aspect of the present invention relates to a method of making a recombinant factor VIII having increased specific activity compared to that of a wild-type factor VIII. This method involves altering the amino acid sequence of a wild-type factor VIII to yield a recombinant factor VIII. Alteration of the amino acid sequence of the wild-type factor VIII can include, for example, introducing at least one point mutation in

or near at least one calcium binding site of the wild-type factor VIII. Thereafter, using protein analysis techniques well-known in the art, a determination can be made as to whether the recombinant factor VIII has increased specific activity compared to that of the wild-type factor VIII.

Another aspect of the present invention relates to a method of treating an animal for hemophilia A. This method involves administering to an animal exhibiting hemophilia A an effective amount of the recombinant factor VIII of the present invention, whereby the animal exhibits effective blood clotting following vascular injury. A suitable effective amount of the recombinant factor VIII can include, without limitation, between about 10 to about 50 units/kg body weight of the animal. The animal can be any mammal, but preferably a human, a rat, a mouse, a guinea pig, a dog, a cat, a monkey, a chimpanzee, an orangutan, a cow, a horse, a sheep, a pig, a goat, or a rabbit.

The recombinant factor VIII of the present invention can be used to treat uncontrolled bleeding due to factor VIII deficiency (e.g., intraarticular, intracranial, or gastrointestinal hemorrhage) in hemophiliacs with and without inhibitory antibodies and in patients with acquired factor VIII deficiency due to the development of inhibitory antibodies. In a particular embodiment, the recombinant factor VIII, alone, or in the form of a pharmaceutical composition (i.e., in combination with stabilizers, delivery vehicles, and/or carriers) is infused into patients intravenously according to the same procedure that is used for infusion of human or animal factor VIII.

Alternatively, or in addition thereto, the recombinant factor VIII can be administered by administering a viral vector such as an adeno-associated virus (Gnatenko et al., *Br. J. Haematol.* 104:27-36 (1999), which is hereby incorporated by reference in its entirety), or by transplanting cells genetically engineered to produce the recombinant factor VIII, typically *via* implantation of a device containing such cells. Such transplantation typically involves using recombinant dermal fibroblasts, a non-viral approach (Roth et al., *New Engl. J. Med.* 344:1735-1742 (2001), which is hereby incorporated by reference in its entirety).

The treatment dosages of recombinant factor VIII that should be administered to a patient in need of such treatment will vary depending on the severity of

the factor VIII deficiency. Generally, dosage level is adjusted in frequency, duration, and units in keeping with the severity and duration of each patient's bleeding episode. Accordingly, the recombinant factor VIII is included in a pharmaceutically acceptable carrier, delivery vehicle, or stabilizer in an amount sufficient to deliver to a patient a therapeutically effective amount of the protein to stop bleeding, as measured by standard clotting assays.

Factor VIII is classically defined as that substance present in normal blood plasma that corrects the clotting defect in plasma derived from individuals with hemophilia A. The coagulant activity *in vitro* of purified and partially-purified forms of factor VIII is used to calculate the dose of recombinant factor VIII for infusions in human patients and is a reliable indicator of activity recovered from patient plasma and of correction of the *in vivo* bleeding defect. There are no reported discrepancies between standard assay of novel factor VIII molecules *in vitro* and their behavior in the dog infusion model or in human patients, according to Lusher, J. M. et al. 328 *New Engl. J. Med.* 328:453-459; Pittman, D. D. et al. (1992) *Blood* 79:389-397; and Brinkhous et al. *Proc. Natl. Acad. Sci.* 82:8752-8755 (1985), which are hereby incorporated by reference in their entirety.

Usually, the desired plasma factor VIII activity level to be achieved in the patient through administration of the recombinant factor VIII is in the range of 30-100% of normal. In one embodiment, administration of the therapeutic recombinant factor VIII is given intravenously at a preferred dosage in the range from about 5 to 50 units/kg body weight, and particularly in a range of 10-50 units/kg body weight, and further particularly at a dosage of 20-40 units/kg body weight; the interval frequency is in the range from about 8 to 24 hours (in severely affected hemophiliacs); and the duration of treatment in days is in the range from 1 ~~to 10 days or until~~ the bleeding episode is resolved. See, e.g., Roberts, H. R., and M. R. Jones, "Hemophilia and Related Conditions--Congenital Deficiencies of Prothrombin (Factor II, Factor V, and Factors VII to XII)," Ch. 153, 1453-1474, 1460, in *Hematology*, Williams, W. J., et al., ed. (1990), which is hereby incorporated by reference in its entirety. Patients with inhibitors may require a different amount of recombinant factor VIII than their previous form of factor VIII. For example,

patients may require less recombinant factor VIII because of its higher specific activity than the wild-type VIII and its decreased antibody reactivity. As in treatment with human or plasma-derived factor VIII, the amount of therapeutic recombinant factor VIII infused is defined by the one-stage factor VIII coagulation assay and, in selected instances, *in vivo* recovery is determined by measuring the factor VIII in the patient's plasma after infusion. It is to be understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed recombinant factor VIII.

Treatment can take the form of a single intravenous administration of the recombinant factor VIII or periodic or continuous administration over an extended period of time, as required. Alternatively, therapeutic recombinant factor VIII can be administered subcutaneously or orally with liposomes in one or several doses at varying intervals of time.

The recombinant factor VIII can also be used to treat uncontrolled bleeding due to factor VIII deficiency in hemophiliacs who have developed antibodies to human factor VIII.

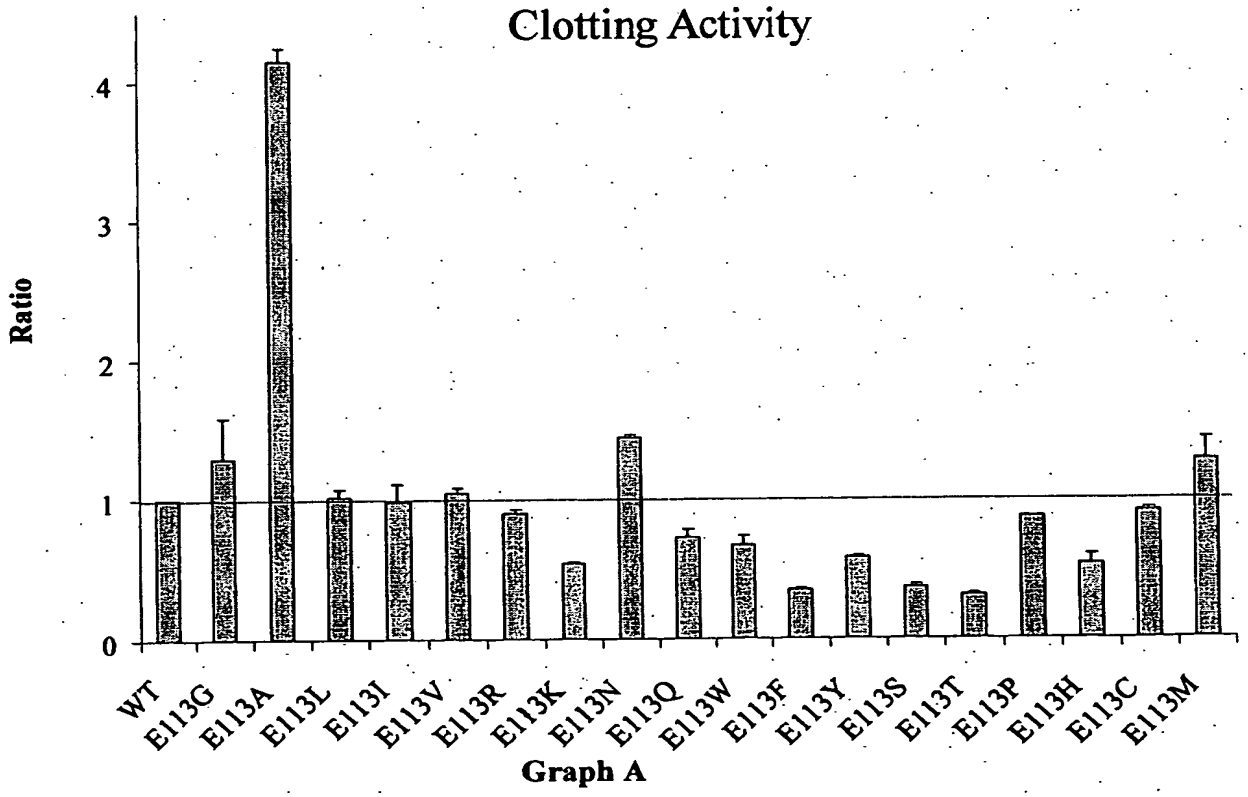
It has been demonstrated herein that the recombinant factor VIII of the present invention can differ in specific activity from the wild-type factor VIII. Factor VIII proteins having greater procoagulant activity from wild-type factor VIII are useful in treatment of hemophilia because lower dosages will be required to correct a patient's factor VIII deficiency. This will not only reduce medical expense for both the patient and the insurer, but also reduce the likelihood of developing an immune response to the factor VIII (because less antigen is administered).

EXAMPLES

The following disclosure is provided to illustrate embodiments of the present invention but are by no means intended to limit its scope.

Clotting Activity Following Saturation Mutagenesis at E113 of the Wild-Type Human Factor VIII:

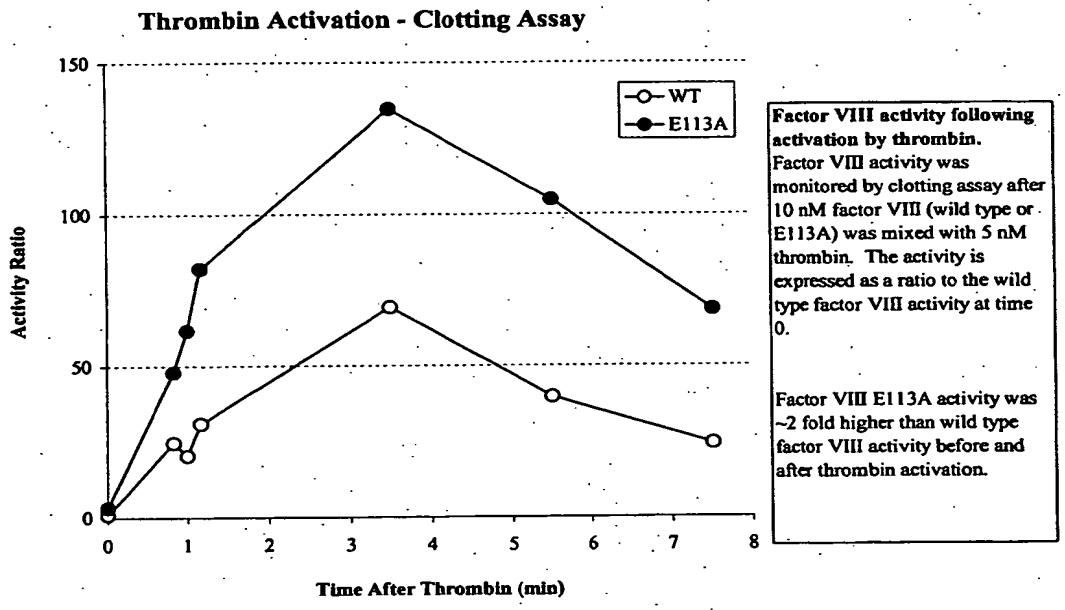
Factor VIII molecules bearing the indicated (see Graph A, below) single point mutations at residue 113 were constructed according to the method described below. The factor VIII expression vector constructs (HSQ-MSAB-NotI-RENeo) were transfected into confluent Cos-7 cells using FuGene6 (Roche, Indianapolis, IN). After 1 day, the medium was changed to AIM-V (Invitrogen) and cultured for an additional 2 days. Conditioned medium containing the expressed factor VIII was collected and factor VIII activity was measured using a one-stage clotting assay. Activity is presented relative to a transfected wild-type control representing a value of (1).



Graph A

Results from this analysis show that mutant E113A possesses significantly greater clotting activity than that observed for the wild-type protein. Furthermore, several other point mutations at this position, including E133L, E113I, E113V, E113N, E113G and E113M show similar or modestly greater clotting activity compared with wild-type.

The clotting activity of the thrombin-activated factor VIII mutant E113A is shown in the figure below. The data below and elsewhere herein demonstrate that both factor VIII and factor VIIIa forms of the mutant demonstrate an ~2-fold increased activity.



The following examples describe the mutagenesis of the amino acid sequence at positions 110-126 of the factor VIII, the measurement of Ca^{2+} and Mn^{2+} binding affinities of the recombinant mutant factor VIII, the measurement of the pro-coagulant activity of the recombinant factor VIII, and the construction of expression vectors that encode the recombinant factor VIII.

INTRODUCTION

Factor VIII, a plasma protein that participates in the blood coagulation cascade, is decreased or defective in individuals with hemophilia A. Factor VIII functions as a cofactor for the serine protease factor IXa in the surface-dependent conversion of zymogen factor X to the serine protease, factor Xa (1,2). Deficiency of factor VIII activity results in a marked reduction of factor IXa activity and in the subsequent rates of factor Xa generated during the propagation phase of coagulation.

Factor VIII is synthesized as an ~300-kDa single chain precursor protein (3,4) with domain structure A1-A2-B-A3-C1-C2 (5). Factor VIII is processed to a series of divalent metal ion-linked heterodimers (6-8) by cleavage at the B-A3 junction, generating a heavy chain (HC^1) minimally represented by the Al-A2 domains; and a light chain (LC) consisting of the A3-C1-C2 domains. The A domains of factor VIII share homology with the A domains of factor V and the copper-binding protein, ceruloplasmin (9). One mol of copper has been identified in factor VIII (10,11).

Metal ions play an important role in regulating factor VIII structure and activity. Factor VIII is inactivated by EDTA, which facilitates dissociation of the HC and LC (6,8). Active factor VIII can be reconstituted by combining the isolated subunits in the presence of Ca^{2+} or Mn^{2+} (12-14). In addition, the presence of low levels of Cu^{+} or Cu^{2+} stimulates this effect (11,15). Thus, it was thought that the linkage of HC and LC by a metal ion (Ca^{2+}, Mn^{2+}, or Cu^{2+}) formed an active heterodimer. This interpretation was consistent with studies examining

the reconstitution of factor Va from isolated subunits by Ca^{2+} or Mn^{2+} (16,17). We recently evaluated the metal ion-dependent and independent association of factor VIII chains (18). In the absence of metal ions, LC and HC combine to form an inactive heterodimer as demonstrated by fluorescence energy transfer. Ca^{2+} had little effect on inter-subunit affinity yet converted the inactive dimer to an active, although low specific activity form. Alternatively, Cu^{2+} enhanced the inter-subunit affinity ~100-fold but yielded a dimer lacking cofactor activity. However, the presence of both metal ions resulted in a high inter-subunit affinity and yielded high specific activity factor VIII. A recent study on the role of Ca^{2+} in factor VIII indicated that Ca^{2+} binding to both factor VIII chains was required for the generation of cofactor activity (19). These studies also demonstrated a local conformational change in response to Ca^{2+} binding correlated with formation of the active cofactor. Factor VIII activity is also reconstituted following addition of Mn^{2+} (12). However, the Mn^{2+}-binding site appeared non-identical to the Ca^{2+} binding site based upon phosphorescence studies showing differential competition by Tb^{3+} (20).

Ca^{2+}-binding proteins typically coordinate Ca^{2+} by 6 to 8 ligands comprised of side chain carboxyl oxygen of acidic amino acids and carbonyl oxygen from the peptide bond (21). While Ca^{2+} binding motifs have been identified, many Ca^{2+} binding proteins coordinate the ion at sites other than a consensus sequence. Recently it was reported that residues 94-110 in factor V, a region rich in acidic amino acids, bound Ca^{2+}, promoting the association of factor Va HC and LC (22). An homologous region in factor VIII, residues 110-126, contains four Asp and four Glu residues in the 16 residue segment.

In the present study, we show that Ca^{2+} binding to the A1 domain of factor VIII is critical for activity generation and directly demonstrate this binding by ITC using the isolated A1 subunit of factor VIIIa. Site-directed mutagenesis is employed to identify residues important in Ca^{2+} binding to factor VIII and resultant effects of this binding on activity. Results of this study identify several acidic amino acids within segment 110-126 of the A1 domain of factor VIII that likely participate in the coordination of Ca^{2+} necessary for generation of maximal cofactor specific activity. Furthermore, comparative data examining Mn^{2+} binding suggest that this ion-binding site partially overlaps with the Ca^{2+} site. Based upon these data, we propose a model whereby occupancy of the metal ion-binding site stabilizes a conformation between the A1 and C1 domains in the cofactor.

MATERIALS AND METHODS

Reagents - Recombinant factor VIII (Kogenate™) was a gift from Dr. Lisa Regan of Bayer Corporation (Berkeley, CA). Phospholipid vesicles containing 20% PS, 40% PC, and 40% PE were prepared using octylglucoside as described previously (23). The reagents α-thrombin, factor IXaβ, factor X, and factor Xa (Enzyme Research Laboratories, South Bend, IN), hirudin, phospholipids, $MnCl_2$ (Sigma, St. Louis, MO), and the chromogenic Xa substrate S-2765 (N-α-benzyloxycarbonyl-D-arginyl-glycyl-L-arginyl-*p*-nitroanilide-dihydrochloride; DiaPharma, West Chester, OH) were purchased from the indicated vendors. The B domainless factor VIII (FVIIIHSQ) expression construct HSQ-MSAB-NotI-RENeo was a gift kindly provided by Dr. Pete Lollar and John Healy.

Preparation of factor VIII and subunits - Factor VIII LC, HC, A1, and A2 subunits were isolated from factor VIII as previously described (24). Proteins were dialyzed into 10 mM MES, 0.3 M KCl, 0.01% Tween-20, pH 6.5, and stored at -80°C.

Construction, expression and purification of factor VIII mutants- B domainless-factor VIII cDNA was restricted from the factor VIII expression construct HSQ-MSAB-NotI-RENeo, using the endonucleases XhoI and NotI, and cloned into the Bluescript II K/S vector. Factor VIII molecules bearing single point mutation of Glu110Ala, Glu110Asp, Glu113Ala, Asp115Ala, Asp116Ala, Glu122Ala, Glu122Asp, Glu124Ala, Asp125Ala, or Asp126Ala, were constructed. Mutations were introduced into the shuttle constructs using the Stratagene QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as described (25). Upon confirmation of the presence of only the desired mutations by dideoxy-sequencing, the appropriate fragment was restricted and cloned back into the factor VIII expression construct. Presence of only the desired mutations was confirmed by a second round of dideoxy-sequencing (Integrated DNA Technologies, Coralville, IA).

The factor VIII expression vector constructs were transfected in BHK cells using FuGene6 (Roche, Indianapolis, IN). The selection, sub-cloning, and cloning of stable transfectants were performed by standard methods and the cloned cells were cultured in roller bottles (25). The conditioned media was collected daily and the expressed proteins were purified using a one-step chromatography scheme as follows. The conditioned medium (~0.3 L) was centrifuged at 3,000 x g for 20 min and the supernatant was filtered through 0.22 um filter. The pH of the filtrate was adjusted to 6.0 and material was loaded onto a column of SP-sepharose (5

ml; Amersham-Pharmacia) equilibrated with 10 mM MES, pH 6.0, 0.2 M NaCl, 0.01% Tween 20. After washing with 20 mM HEPES, pH 7.2, 0.2 M NaCl, 0.01 % Tween 20, the bound factor VIII was eluted by with 20 mM HEPES, pH 7.2, 0.8 M NaCl, 0.01 % Tween 20. Active fractions were detected using a one-stage clotting assay, pooled and dialyzed against 10 mM MES pH 6.5, 0.3 M KCl, 0.01 % Tween 20 in Chelex100 treated ddH_2O. Resultant factor VIII forms were typically >80% pure as judged by SDS-polyacrylamide gel electrophoresis with albumin representing the major contaminant. Factor VIII samples were quick frozen and stored at -80°C.

Factor Xa Generation Assays –The rate of conversion of factor X to factor Xa was monitored in a purified system (26) according to the method previously described (18,19). Activity was determined as the amount of factor Xa generated (nM) per minute and converted to a value per nM factor VIII.

Preincubation of factor VIII subunits with Ca^{2+} - Mixtures of A1 and A3-C1-C2 (2 μM and 1 μM, respectively) in 10 mM MES, 0.3 M KCl, 0.01% Tween-20, 0.01% BSA, pH 6.5) and A2 (10 μM in 20 mM HEPES, 0.05 M KCl, 0.01% Tween-20, 0.01% BSA, pH 7.2) were separately pre-incubated with 3 mM Ca^{2+} or 0.1 mM EDTA for 18 hour at 4°C. Reactions were initiated by mixing A1/A3-C1-C2 and A2 solutions at a final subunit concentration of 40/20/200 nM (A1/A3-C1-C2/A2) in 20 mM HEPES, 0.05 M KCl, 0.01% Tween 20, 0.01% BSA, pH 7.2 (residual Ca^{2+} and EDTA concentrations were 0.3 mM and 4 μM, respectively). At the indicated times, aliquots were removed and the activity was measured by the factor Xa generation assay.

Isothermal titration calorimetry for Ca^{2+} binding on A1 - ITC was performed to measure Ca^{2+} binding to the isolated A1 subunit using a VP-ITC MicroCalorimetry Systems Instrument (MicroCal, Northampton, MA). The concentration of A1 was determined by A_{280} value using an extinction coefficient = 58,350 $cm^{-1}M^{-1}$ based upon the amino acid sequence for the A1 domain (factor VIII residues 1-372) according to the method of Gill and von Hippel (27). A1 subunit (25.6 μM) was treated with 10 mM EDTA for 18 hours at 4°C, followed by a dialysis against 10 mM MES, pH 6.5, 0.3 M KCl, 0.01% Tween20. The dialysis buffer was made using Chelex 100 treated H_2O and the system was extensively washed with Chelex 100-treated H_2O prior to use. Samples and buffers were degassed prior to analysis. The A1-containing solution was placed in a 1.44 ml sample cell. A 700 μL syringe loaded with 2 mM $CaCl_2$ in the same buffer was used for a series of automatic injections of 2 μL each into the A1 solution while mixing at a rate of 290 rpm at 30 °C. The cumulative total of the heat evolved was plotted against the total Ca^{2+} concentration to produce a binding isotherm. Each injection was followed by a 240 s pause to allow the system to return to a baseline value. Since heat produced from dilution, as measured by injecting the Ca^{2+} solution into the sample cell containing only the buffer, was negligible, the uncorrected data was used for the analysis. An identical independent binding model was fit to the data and thermodynamic parameters [enthalpy (ΔH^0), K_d, and molar binding stoichiometry (n)] were determined by nonlinear least squares regression using the ORIGIN software. Subsequently Gibbs free energy (ΔG) and entropy (ΔS^0) were calculated from the fitted values.

Factor VIII activity titration using Ca^{2+} or Mn^{2+} - Ca^{2+}-EGTA buffer with free Ca^{2+} concentrations of 0- 6.5 mM and Mn^{2+}-EGTA buffer with free Mn^{2+} concentrations of 0-

0.75 mM in the presence of 2 mM EGTA were prepared as previously described (19,20). Wild type or mutant HSQ factor VIII (50 nM) was reacted in the Ca^{2+}-EGTA buffer or Mn^{2+}-EGTA buffer at 4°C for 18 hours and resultant factor VIII activity was measured using the factor Xa generation assay. Non-linear least squares regression analysis was performed according to a single-site binding model using the formula,

$$Activity = \frac{k \cdot [Me^{2+}]}{K_d + [Me^{2+}]} + C$$

where k is constant reflecting the metal ion induced activity , $[Me^{2+}]$ is either free Ca^{2+} or free Mn^{2+} concentration, K_d is the dissociation constant, and C is constant reflecting the basal activity in the absence of exogenous metal ion.

Enzyme-Linked Immunoadsorbant Assay- A sandwich ELISA was preformed to measure the concentration of HSQ factor VIII proteins (25). The procedure employed ESH8 (anti-factor VIII LC antibody; American Diagnostica) as a capture antibody and biotinylated R8B12 (anti-factor VIII A2 antibody; Green Mountain Antibodies) as the detection antibody. Thus, the epitopes for these antibodies are far-removed from the sites of mutagenesis. The amount of bound factor VIII was determined optically using a streptoavidin-linked horse radish peroxidase (Calbiochem) with the substrate, O-phenylenenediamine dihydrochloride, (Calbiochem) as previously described (25). Purified commercial recombinant factor VIII was used as the standard to determine the concentration of the samples. Factor VIII specific activity was determined form one-stage clotting assays and ELISA and is expressed as units/μg.

Statistical Analysis - Nonlinear least-squares regression analysis was performed by Kaleidagraph (Synergy, Reading, PA) to obtain parameter values and standard deviations.

RESULTS

Preincubation of factor VIII subunits with Ca^{2+} or EDTA followed by activity reconstitution

Previously we demonstrated that maximal cofactor activity was achieved only when both HC and LC were pre-incubated with Ca^{2+} (19), suggesting that Ca^{2+} binding to both HC and LC was necessary to generate active factor VIII. A similar evaluation of factor VIIIa reconstitution from the isolated A1, A2, and A3-C1-C2 was performed to determine the Ca^{2+} requirement for the HC-derived A1 and A2 subunits in activity generation. The reconstitution of factor VIIIa is a two-step process with the initial association of A1 and A3-C1-C2 comprising the rate-limiting step and requiring several hours to complete (28). Therefore, we completed this first step by mixing A1 and A3-C1-C2 subunits (2:1, mol:mol) in the presence of either 3 mM Ca^{2+} or 0.1 mM EDTA for 18 hours. Activity generation was then monitored following the addition of A2 subunit, which, like the other subunits, was pre-incubated with either 3 mM Ca^{2+} or 0.1 mM EDTA. The reconstituted A1/A3-C1-C2 dimer and A2 subunit were diluted 50-fold prior to reconstitution to prevent the EDTA-treated component from acquiring Ca^{2+} at the time of reconstitution. Furthermore, the reconstitution time course (30 min) was short enough so that the dissociation of Ca^{2+} from subunits upon their dilution was not a concern. Evaluation of the negative control (both A1/A3-C1-C2 dimer and A2 subunit pre-treated with EDTA) did not generate any activity over the reconstitution time course (Figure 1). On the other hand, recombining the Ca^{2+}-treated A1/A3-C1-C2 dimer and A2 subunit resulted in the rapid

generation of factor VIIIa activity (Figure 1) that reached a maximal level within 10 min. When Ca^{2+}-treated A1/A3-C1-C2 was associated with EDTA-treated A2, the generated activity was similar to the positive control (~90% activity at 10 min and ~80% activity at 30 min). Assuming the association rates for Ca^{2+} binding on each subunit was similar, these data suggested that there was little if any contribution of Ca^{2+} binding to A2 subunit for activity generation. Consistent with this result was the failure to reconstitute factor VIIIa activity with the Ca^{2+}-treated A2 plus EDTA-treated dimer. These results, taken together with our earlier observation on the requirement for Ca^{2+}-binding to HC for efficient factor VIII reconstitution (19) indicates that Ca^{2+} binding to A1 subunit is a prerequisite for activity generation.

Ca^{2+} binding to A1 detected by ITC.

The binding of Ca^{2+} to isolated A1 subunit was directly examined using ITC. Initial Ca^{2+} injections into the A1-containing solution showed a large exothermic peak (Figure 2), providing direct evidence for binding of the metal ion to the factor VIIIa subunit. Data were fitted using an identical independent binding model for cautious interpretation. The apparent thermodynamic values obtained from the binding isotherm were $\Delta H^0 = -4.76 \pm 0.03$ kcal/mole and $K_d = 0.74 \pm 0.05$ μM. ΔS^0 and ΔG values were calculated as 12.3 kcal/mol/K and -8.5 kcal/mol, respectively. Thus ΔH^0 comprised 56% of ΔG, indicating that there was nearly equal contribution of enthalpy and entropy to the free energy change following the binding of Ca^{2+} to the A1 subunit. The observation of a large entropy change upon Ca^{2+} binding to A1 suggested a complex mechanism likely involving a significant conformational component. Interestingly, a stoichiometry of 2.4 was obtained from the fitted data indicating the presence of more than one Ca^{2+} sites contained within the A1 subunit.

Factor VIII mutations of a putative Ca^{2+}-binding site in A1.

The above data indicate the presence of a Ca^{2+} site(s) within the A1 domain of factor VIII that is (are) required for cofactor activity. Based upon the homology of factor VIII residues 110-126 to the residues comprising a putative Ca^{2+}-binding site localized in factor V, we constructed a series of point mutations where acidic residues were replaced with Ala (or in some cases Asp). The stable transfectants were expressed as B-domainless factor VIII in BHK cells and recombinant factor VIII was purified as described in Methods. The freshly purified factor VIII preparations (mutants and wild type) were dialyzed against metal ion-free buffer, and specific activity values were determined by one-stage clotting and sandwich ELISA assays (Table 1). This treatment resulted in the retention of a significant level of activity, as judged by a specific activity of 4.8 units/μg for the wild type factor VIII, while removing exogenous metal ions from the protein preparations. The activity observed under these conditions likely reflected retention of a metal ion(s), possibly Ca^{2+}, which is (are) not readily released in the absence of chelators. This property is not due to the presence of single chain factor VIII (~30-50% of total factor VIII) in the recombinant preparations since partial purification of the factor VIII to enrich for single chain material yielded a similar specific activity as the unfractionated factor VIII preparation (data not shown).

Several of the Ala-substituted point mutations (E110A, D116A, E122A, D125A, and D126A) exhibited marked reductions in specific activity to levels of ~4 to 12% of the wild type value (Table I). Thus the reduction in volume of the side chain and/or loss in electrostatic potential may result in slight conformational changes within this region that impair cofactor

activity. Since results from a prior study evaluating a Ca^{2+} site in lactalbumin showed the importance of side chains when replacing critical residues (29), we made selected, additional mutants with the conservative substitution of Asp for Glu at residues 110 and 122. As shown in Table I, significantly greater activity was retained in the E110D and E122D mutants (10.1 and 22.4%, respectively) compared with E110A and E122A mutants (3.8 and 12.2%, respectively)

Cofactor activity generated from factor VIII mutants following titration with Ca^{2+}

Our prior studies examining Ca^{2+} binding in factor VIII employed isolated HC and LC prepared from the EDTA-treated heterodimer (18,19). Mixing of chains in the absence of Ca^{2+} resulted in no regenerated activity. In the current study, limitations in the amounts of mutant factor VIII precluded chain separation and purification. However, we observed that the basal activity of the factor VIII measured in the absence of exogenous metal could be increased ~2-3-fold with saturating levels of Ca^{2+}. This incremental activity increase provided a functional assay for the binding of Ca^{2+} to the factor VIII A1 domain mutants.

Increases in cofactor activity for the factor VIII wild type and 110-126 mutants in the absence of exogenous metal ion was determined following titration with Ca^{2+}. Results are presented in Figure 3 and are arbitrarily divided into high, moderate and low activity factor VIII forms. Estimated parameter values determined by nonlinear least-squares curve fitting are listed in Table II. An optimized range of Ca^{2+} concentrations (0-6.5 mM) was selected to cover the complete change in activity for all factor VIII forms. We observed no significant increase in activity at higher concentrations of Ca^{2+} (>10 mM; data not shown). The k value indicates the difference between maximum activity at the saturation with Ca^{2+} and the minimum activity with

no exogenous metal ion present (C value). Therefore, the k value was used as an indicator to assess the activity response for each mutant to added Ca^{2+}.

Wild type factor VIII and many factor VIII mutants displayed an increase in activity in response to increases in the concentration of Ca^{2+}. Maximal activity response for the wild type reflected a high affinity for Ca^{2+} (K_d = 1.18 μM, Table II-1) and this value compared favorably with a K_d = 8.9 μM for Ca^{2+} binding as measured in a functional assay for reconstituted factor VIII HC and LC (19), as well as with the value determined above from ITC analysis of the isolated A1 subunit. Two mutations (E113A and E122D) showed little deviation from the wild-type affinity parameters. On the other hand, four of the factor VIII mutants tested, E110D, D116A, E122A, and D126A showed ~25-90-fold increases in K_d for Ca^{2+} binding compared to wild type, indicating a marked reduction in affinity for the metal ion and suggesting a possible role for these residues in forming a Ca^{2+} binding site. Comparison of the results obtained for E122D and E122A showing an ~3- and ~30-fold reduction in Ca^{2+} affinity suggested the conserved substitution was relatively benign compared with the Ala substitution. A similar disparity was observed for mutation at E110 where the Asp substitution yielded an ~25-fold reduction in affinity while substitution with Ala appeared to eliminate the Ca^{2+} binding site. These results suggested a significant role for these residues, especially E110, in Ca^{2+} binding. The loss of Ca^{2+} binding was also observed with mutation at D125. Based upon the observed defects in Ca^{2+} binding and/or affinity, we propose that residues E110, D116, E122, D125 and D126 form a Ca^{2+}-coordination site in the A1 domain of factor VIII. We also speculate that E110 and D125 are critical to this site since alteration of these residues appeared to result in loss of Ca^{2+} binding. Furthermore, we suggest that residues D115 and E124 make little contribution

to Ca^{2+} coordination. The basis for this contention is the minimal effect of Ala substitution on Ca^{2+} binding at these sites, inasmuch as K_d values were increased by <9-fold. This modest reduction in affinity may arise from Ala substitution at these residues affecting the contributions of the adjacent residues D116 and D125, respectively to the Ca^{2+}-binding site.

Cofactor activity generated from factor VIII mutants following titration with Mn^{2+}

In a recent report, we showed that Mn^{2+} binds factor VIII with high affinity (5.7 μM) and results in similar stimulation of cofactor activity (20). However, that study also revealed competition of Tb^{3+} binding to factor VIII by Mn^{2+} but not by Ca^{2+}, indicating that the Mn^{2+} and Ca^{2+} binding sites in factor VIII were not identical. In order to determine whether any of the residues we identify above as participating in binding Ca^{2+} contribute to forming a Mn^{2+}-binding site, a similar approach was employed where factor VIII activity was measured in response to titration with Mn^{2+}. Results of these studies are shown in Figure 4 and Table II-2, and employed a range of Mn^{2+} concentrations from 0-0.75 mM (concentrations >5 mM resulted in no further increase in activity, data not shown). Several parallels in the response to Ca^{2+} were observed using Mn^{2+}. Wild type factor VIII displayed a high affinity for Mn^{2+} (K_d = 1.40 μM). Most of the mutants showed an increase in activity following addition of Mn^{2+}, and activity values at saturating concentration of Mn^{2+} (k values) were very similar to those observed for Ca^{2+}. Thus the value for the activity response varied depending upon the particular mutation rather than the metal ion used to saturate the response, suggesting that the activity response could result from modest changes in conformation that were unrelated to the specific metal-ion binding event. Therefore, with respect to this particular site in the A1 domain, both Ca^{2+} and Mn^{2+} generate activity by a mechanism affecting a common region crucial for cofactor function.

In contrast, while we observed markedly reduced Ca^{2+} affinities for E122A and D126A, the affinity of these factor VIII mutations for Mn^{2+} was either only marginally (~2-fold) reduced or unchanged, respectively. We did observe an ~8-fold reduction in Mn^{2+} for the mutant D116A (compared with a ~40-fold reduction in Ca^{2+} affinity), and this result may suggest a role for D116 in the coordination of Mn^{2+}. Interestingly, the two mutations that showed little if any response to Ca^{2+} (E110A and D125A) were also unresponsive to Mn^{2+}. Substitution of Asp for Glu at residue 110 partially restored Ca^{2+}-dependent function but had little effect on the Mn^{2+}-dependent activity, suggesting that this residue does not likely function in binding Mn^{2+}. While mutations at E110 showed marginal activity relative to wild type in the absence of exogenous metal ion (C = 3.2% and 7.2 % for Ala and Asp substitutions), the mutation D125A retained significant activity (C = 41%). This observation indicated that mutation at D125 did not likely result in any global change in conformation that would diminish factor VIII activity. This observation adds strong support to our conclusion that D125 participates in the coordination of either Ca^{2+} or Mn^{2+}.

DISCUSSION

Previously, we found that Ca^{2+} (or Mn^{2+}) binding to factor VIII HC was essential for cofactor activity (19,20). We now identify and tentatively localize a Ca^{2+}-binding site in the A1 domain of factor VIII, the occupancy of which yields an increase in specific activity. Furthermore, the observation that Ca^{2+} binding to A2 domain in HC contributes little if at all to generate cofactor activity highlights the functional role of the Ca^{2+} binding site in A1 domain in

HC. Recently Zeibdawi *et al.* (22) reported that residues 94-110 in factor V comprise a Ca^{2+} binding site required for its activity. In the current study, we probed the homologous region in the A1 domain of factor VIII (residues 110-126) for Ca^{2+} binding using a site-directed mutagenesis approach. Our results show that mutation at each of several acidic amino acids (E110, D116, E122, D125, and D126) caused a marked reduction (or complete loss) of Ca^{2+} binding affinity, providing evidence that these residues participate in coordinating Ca^{2+}. In addition, data from a complementary study revealed that in the absence of Ca^{2+}, D125 (and possibly D116) likely contribute to the coordination of Mn^{2+}. Thus, these results are consistent with an earlier report showing that Ca^{2+} and Mn^{2+} bind to non-identical sites in HC (20) and further suggest that these sites are in close proximity to one-another.

Mechanism(s) by which Ca^{2+} (or Mn^{2+}) generate active factor VIII remain largely unknown. The factor VIII A domain homology model (30) predicts residues 102-116 not to possess a defined secondary structure while residues 120-125 form an α-helix with a short β strand segment (residues 117-119) connecting the two segments (Figure 5). Based upon the results in the present study, we propose that Ca^{2+} stabilizes this region by forming bonds with E110, D116, E122, D125, and/or D126. This coordination would provide appropriate energy to fix in space the elongated region defined by 110-116. Furthermore, it is of interest to note that in the 5-domainal factor VIII model (31), this region juxtaposes the C1 domain (see Figure 5). While A1 and A3 domains appear to associate with a relatively extended interface, the interface between A1 and C1 is small. Thus we speculate that stabilizing a segment in A1 near C1 may add structure to a "hinge" region separating the A and C domains.

The above hypothesis is reinforced by our results obtained with Mn^{2+}, which is typically coordinated by acidic residues and/or His residues (32). There are two His residues in C1 (H2082 and H2137) that are in close proximity to residues 110-126 in A1. We propose that these His residues contribute to Mn^{2+} coordination with D125 (and possibly D116). The result of this coordination could also stabilize the interaction of A1 and C1 by bridging these regions. This explanation is compatible with our results that Ca^{2+} and Mn^{2+} bind different sites (20) yet generate active factor VIII of similar specific activity. Furthermore, this hypothesis also offers an explanation for the increase in Mn^{2+} affinity observed for several of the A1 mutants. Thus some mutations may have resulted in an altered spatial separation between D125 (and D116) and His residue(s) H2082 and/or H2137 in C1 and this alteration may be favorable for Mn^{2+} coordination, yielding a higher affinity for the metal ion. This hypothesis is compatible with preliminary data suggesting that the effects of Ca^{2+} and Mn^{2+} on factor VIII activity generation are neither additive nor synergistic (data not shown).

Overall, the stabilization we propose to result from metal ion binding near the A1-C1 junction may be necessary to provide proper orientation of factor VIIIa subunits within the factor Xase complex. Significant data indicate an extended interface between factor VIIIa and factor IXa, mediated by residues in A2 and A3 domains of the cofactor (see Ref. (33) for review). While residues in A3 appear to provide the majority of the binding energy for this interaction (34), critical contacts between A2 subunit and the protease domain of factor IXa are required for cofactor function (35). The latter is borne-out by the direct stimulation of factor IXa by the isolated A2 subunit (36). While A1 subunit does not appear to contact factor IXa directly, inclusion of isolated A1 subunit results in a marked enhancement of the activity attributed to the

isolated A2 subunit (37). Thus A1 appears to function to orient A2 relative to the factor IXa protease domain. This property if further illustrated by truncation of A1 at R336 resulting in a dramatic loss in cofactor activity without significantly altering the inter-A1-A2 subunit affinity (38).

Factor VIII HC and LC associate in the absence of metal ion with moderate affinity (K_d = 53.8 ± 14.2 nM) (18) and inclusion of either Ca^{2+} or Mn^{2+} did not change the affinity of this interaction (K_d = 48.7 ± 15.4 (19) and 53.0 ± 17.1 nM (20) in the presence of Ca^{2+} and Mn^{2+}, respectively). Thus the binding energy for interaction of HC and LC is likely derived from electrostatic and hydrophobic interactions between A1 and A3 domains. As described above, Ca^{2+} or Mn^{2+} binding the A1-C1 boundary region may create a fractional contribution to the total binding energy between HC and LC and thus remain undetected in the inter-chain affinity determination. Analysis of the kinetics of factor VIII activity generation of the HC/LC complex, associated in the absence of metal ions, following addition of Ca^{2+} yielded a series reaction pattern, suggesting that Ca^{2+} binding triggers certain conformational change(s) within the heterodimer to yield active factor VIII (19). Conformational events suggested by the current study may reflect the stabilization of the A1 110-126 region, followed by formation of a stable interface between this region and the region around H2137 in the C1 domain.

We identified the presence of at least two Ca^{2+} sites in isolated A1 subunit by ITC following its treatment with EDTA. The large enthalpy change observed upon binding Ca^{2+} was consistent with a significant change in conformation of this domain as suggested above. The affinity value measured for the sites (~0.7 μM) was similar to the value we obtained monitoring

the increase in specific activity (1.18 μM for B-domain less wild type factor VIII). Furthermore, the fractional stoichiometry observed for occupancy of the isolated domain may suggest a dimerization of the subunit that is not observed with the intact heterodimer. The relationship of Ca^{2+} sites in the A1 domain with other sites in factor VIII has not yet been established. While we observed passive removal of a putative Ca^{2+} molecule(s) from the site we propose within residues 110-126, other metal ions likely remain associated as judged by the relatively high specific activity of the protein in solutions free from exogenous metal ions. Based upon the observation that pre-treatment of EDTA-treated factor VIII LC with Ca^{2+} was required to obtain reconstitution of functional factor VIII (19), we speculate that Ca^{2+} contained within sites in the LC may be retained in the absence of chelation. In support of this contention, preliminary data by ITC suggests the presence of multiple Ca^{2+} sites in the factor VIII LC (Wakabayashi and Fay, unpublished observation)

Several drawbacks to a loss-of-function mutagenesis approach in the localization of Ca^{2+}-binding sites have been noted. These include mutation to an Ala eliminating total Ca^{2+} binding (29), or the elimination of charged residues far removed from a Ca^{2+}-biding site (39,40) that result in reduced Ca^{2+} affinity. However, the results presented in this report are further supported by a recent, similar approach applied to the Ca^{2+}-biding site in factor V. The region comprised of residues 110-126 in factor VIII is highly homologous to residues 96-112 in factor V (Figure 6). Recent data generated following site-directed mutagenesis within this region indicates that E96, D102, and D111 appear to be crucial residues for the association of factor Va HC and LC (41), an interaction that is Ca^{2+}-dependent in factor Va (17). Our results indicating a role for factor VIII residues E110, D116 and D126 in Ca^{2+} binding correspond to factor V

residues E96, D102, and D111, respectively. These residues are conserved in all species of factor V and factor VIII identified to date. In addition, we report no role for residues E113, D115, and E124 in Ca^{2+} coordination, and these residues are not conserved in factor V. Thus the identification of selected, homologous residues as determined in two independent studies provides mutual support for the role of this region in contributing to Ca^{2+}-coordination sites in the protein cofactors.

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REFERENCES

1. Davie, E. W. (1995) *Thrombosis & Haemostasis* 74, 1-6

2. Lollar, P. (1995) *Advances in Experimental Medicine & Biology* 386, 3-17

3. Wood, W. I., Capon, D. J., Simonsen, C. C., Eaton, D. L., Gitschier, J., Keyt, B., Seeburg, P. H., Smith, D. H., Hollingshead, P., Wion, K. L., Delwart, E., Tuddenham, E. D. G., Vehar, G. A., and Lawn, R. M. (1984) *Nature* 312, 330-37

4. Toole, J. J., Knopf, J. L., Wozney, J. M., Sultzman, L. A., Buecker, J. L., Pittman, D. D., Kaufman, R. J., Brown, E., Shoemaker, C., Orr, E. C., Amphlett, G. W., Foster, W. B., Coe, M. L., Knutson, G. J., Fass, D. N., and Hewick, R. M. (1984) *Nature* 312, 342-47

5. Vehar, G. A., Keyt, B., Eaton, D., Rodriguez, H., O'Brien, D. P., Rotblat, F., Oppermann, H., Keck, R., Wood, W. I., Harkins, R. N., Tuddenham, E. G. D., Lawn, R. M., and Capon, D. J. (1984) *Nature* 312, 337-42

6. Fass, D. N., Knutson, G. J., and Katzmann, J. A. (1982) *Blood* 59, 594-600

7. Andersson, L. O., Forsman, N., Huang, K., Larsen, K., Lundin, A., Pavlu, B., Sandberg, H., Sewerin, K., and Smart, J. (1986) *Proceedings of the National Academy of Sciences of the United States of America* 83, 2979-83

8. Fay, P. J., Anderson, M. T., Chavin, S. I., and Marder, V. J. (1986) *Biochimica et Biophysica Acta* 871, 268-78

9. Church, W. R., Jernigan, R. L., Toole, J., Hewick, R. M., Knopf, J., Knutson, G. J., Nesheim, M. E., Mann, K. G., and Fass, D. N. (1984) *Proceedings of the National Academy of Sciences of the United States of America* 81, 6934-7

10. Bihoreau, N., Pin, S., Kersabiec, A. M. D., Vodot, F., and Fontaine-Aupart, M. P. (1994) *Eur. J. Biochem.* 220, 41-48

11. Tagliavacca, L., Moon, N., Dunham, W. R., and Kaufman, R. J. (1997) *Journal of Biological Chemistry* 272, 27428-34

12. Fay, P. J. (1988) *Archives of Biochemistry & Biophysics* 262, 525-31

13. Nordfang, O., and Ezban, M. (1988) *Journal of Biological Chemistry* 263, 1115-8

14. Fay, P. J., and Smudzin, T. M. (1989) *Journal of Biological Chemistry* 264, 14005-10

15. Sudhakar, K., and Fay, P. J. (1998) *Biochemistry* 37, 6874-82

16. Esmon, C. T. (1979) *Journal of Biological Chemistry* 254, 964-73

17. Krishnaswamy, S., Russell, G. D., and Mann, K. G. (1989) *Journal of Biological Chemistry* 264, 3160-8

18. Wakabayashi, H., Koszelak, M. E., Mastri, M., and Fay, P. J. (2001) *Biochemistry* 40, 10293-10300

19. Wakabayashi, H., Schmidt, K. M., and Fay, P. J. (2002) *Biochemistry.* 41, 8485-92

20. Wakabayashi, H., Zhen, Z., Schmidt, K. M., and Fay, P. J. (2003) *Biochemistry.* 42, 145-53

21. Means, A. R. (1995) *Adv. Second Messenger Phospho. Res.* 30, 89-151

22. Zeibdawi, A. R., and Pryzdial, E. L. (2001) *Journal of Biological Chemistry* 276, 19929-36

23. Mimms, L. T., Zampighi, G., Nozaki, Y., Tanford, C., and Reynolds, J. A. (1981) *Biochemistry* 20, 833-840

24. Fay, P. J., Mastri, M., Koszelak, M. E., and Wakabayashi, H. (2001) *Journal of Biological Chemistry* 276, 12434-9

25. Jenkins, P. V., Freas, J., Schmidt, K. M., Zhou, Q., and Fay, P. J. (2002) *Blood.* 100, 501-8

26. Lollar, P., Fay, P. J., and Fass, D. N. (1993) *Methods in Enzymology* 222, 128-43

27. Gill, S. C., and von Hippel, P. H. (1989) *Analytical Biochemistry.* 182, 319-26

28. Regan, L. M., and Fay, P. J. (1995) *Journal of Biological Chemistry* 270, 8546-52

29. Anderson, P. J., Brooks, C. L., and Berliner, L. J. (1997) *Biochemistry.* 36, 11648-54

30. Pemberton, S., Lindley, P., Zaitsev, V., Card, G., Tuddenham, E. G., and Kemball-Cook, G. (1997) *Blood* 89, 2413-21

31. Stoilova-McPhie, S., Villoutreix, B. O., Mertens, K., Kemball-Cook, G., and Holzenburg, A. (2002) *Blood.* 99, 1215-23

32. Weatherburn, D. C. (2001) (Bertini, I., Sigel, A., and Sigel, H., eds), pp. 193-268, Marcel Dekker, Inc

33. Mertens, K., Celie, P. H., Kolkman, J. A., and Lenting, P. J. (1999) *Thrombosis & Haemostasis* 82, 209-17

34. Lenting, P. J., Donath, M. J., van Mourik, J. A., and Mertens, K. (1994) *Journal of Biological Chemistry* 269, 7150-5

35. Bajaj, S. P., Schmidt, A. E., Mathur, A., Padmanabhan, K., Zhong, D., Mastri, M., and Fay, P. J. (2001) *Journal of Biological Chemistry* 276, 16302-9

36. Fay, P. J., and Koshibu, K. (1998) *Journal of Biological Chemistry* 273, 19049-19054

37. Fay, P. J., Koshibu, K., and Mastri, M. (1999) *Journal of Biological Chemistry* 274, 15401-6

38. Koszelak Rosenblum, M. E., Schmidt, K., Freas, J., Mastri, M., and Fay, P. J. (2002) *Journal of Biological Chemistry.* 277, 11664-9

39. Trigo-Gonzalez, G., Awang, G., Racher, K., Neden, K., and Borgford, T. (1993) *Biochemistry.* 32, 9826-31

40. Ababou, A., Shenvi, R. A., and Desjarlais, J. R. (2001) *Biochemistry.* 40, 12719-26

41. Zeibdawi, A. R., Grundy, J. E., Lasia, B., and Pryzdial, E. L. (2003) *Biochem. J.* in press, Immediate Publication 1 Sept. 2003 on the worldwide web.

Each of the references 1-41 is hereby incorporated by reference in its entirety.

Footnotes

[1]The abbreviations used are: HC, factor VIII heavy chain; LC, factor VIII light chain; EDTA, ethylenediamine tetraacetic acid; ITC, isothermal titration calorimetry; MES, 2-[N-morpholino]ethanesulfonic acid; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]; BSA, bovine serum albumin; PS, phosphatidylserine, PC, phosphatidylcholine; and PE, phosphatidylethanolamine.

FIGURE LEGENDS

Figure 1. Effect of pre-incubation with Ca^{2+} on factor VIII reconstitution from isolated subunits. Factor VIII subunits (A1/A3-C1-C2 and A2) were separately pre-incubated with 3 mM Ca^{2+} or 0.1 mM EDTA for 18 hour. After mixing the pre-incubated A1/A3-C1-C2 and A2, reconstituted factor VIII activity was measured by a factor Xa generation assay as described in Materials and Methods. Mixtures were A1/A3-C1-C2 pre-incubated with Ca^{2+} plus A2 pre-incubated with Ca^{2+} (closed circles), A1/A3-C1-C2 pre-incubated with EDTA plus A2 pre-incubated with Ca^{2+} (squares), A1/A3-C1-C2 pre-incubated with Ca^{2+} plus A2 pre-incubated with EDTA (triangles), and A1/A3-C1-C2 pre-incubated with EDTA plus A2 pre-incubated EDTA (open circles). Each point represents the average of four determinations.

Figure 2. Isothermal titration calorimetry of Ca^{2+} binding to the A1 subunit at 30°C. The top panel shows the heat signal for 30 injections of 2 μL aliquots of 2 mM Ca^{2+} into a 1.44 ml cell containing 25.6 μM A1. Both Ca^{2+} and A1 were in 10 mM MES, pH 6.5, 0.3 M KCl, 0.01% Tween 20. The bottom panel shows the integrated heat for each injection after normalization to the amount of Ca^{2+} added. Lines were drawn from the curve fit using Origin software. The apparent thermodynamic parameters describing the fit are $n = 2.40 \pm 0.01$, $K_d = 0.74 \pm 0.05$ μM, and $\Delta H^0 = -4.76 \pm 0.03$ kcal/mol. ΔS^0 was subsequently calculated as 12.3 kcal/mol/K.

Figure 3. Factor VIII activity following titration with Ca^{2+}. B-domainless-factor VIII forms (50 nM) in the presence of the indicated amounts of free Ca^{2+} with 2 mM EGTA were incubated for 18 hours at 4°C and the factor VIII activity measured by a factor Xa generation assay as

described in Materials and Methods. Each point represents the average of four determinations. (A) High activity species include wild type (open circles), E113A (open triangles), and E115A (open squares). (B) Moderate activity species include E122A (open circles), E122D (open triangles), E124A (open squares), and D126A (closed circles). (C) Low activity species include E110A (open circles), E110D (open triangles), D116A (open squares), and D125A (closed circles). Lines were drawn from the curve fit according to a single-site binding model as described in Materials and Methods.

Figure 4. Factor VIII activity following titration with Mn^{2+}. B-domainless factor VIII forms (50 nM) in the presence of the indicated amounts of free Mn^{2+} with 2 mM EGTA were assessed as described in the legend to Figure 3. (A) High activity species include wild type (open circles), E113A (open triangles), and E115A (open squares). (B) Moderate activity species include E122A (open circles), E122D (open triangles), E124A (open squares), and D126A (closed circles). (C) Low activity species include E110A (open circles), E110D (open triangles), D116A (open squares), and D125A (closed circles). Lines were drawn from the curve fit according to a single-site binding model as described in Materials and Methods.

Figure 5. Structure in and around factor VIII residues 110-126. Factor VIII subunits A1 (light blue), A2 (blue), A3 (light yellow), C1(pink), and C2 (grey) were drawn using an atomic surface model. The region indicated by the white arrow in panel A was magnified and is shown in panels B and C at slightly different angles. The region 102-126 was drawn in ribbon format with the designations: red (α-helix), green (β-strand), and white (loop). The region 2133-2140 in

the C1 domain was drawn using a stick model format without side chains (red-oxygen, blue-nitrogen, and white-carbon).

Figure 6. Sequence alignments of human factor V and human factor VIII. Residues are indicated by the single letter designation. Acidic residues are in bold typeface. Matched acidic residues are underlined.

Table I. Specific Activity of Factor VIII Wild Type and Mutants Forms

	Specific Activity	
Wild Type	4.77 ± 0.54[a]	(100.0[b])
E110A	0.18 ± 0.03	(3.8)
E110D	0.48 ± 0.09	(10.1)
E113A	9.78 ± 0.03	(205.0)
D115A	5.04 ± 0.49	(105.5)
D116A	0.54 ± 0.02	(11.3)
E122A	0.58 ± 0.01	(12.2)
E122D	1.07 ± 0.24	(22.4)
E124A	2.11 ± 0.10	(44.3)
D125A	0.46 ± 0.01	(9.6)
D126A	0.59 ± 0.13	(12.5)

The activity and the concentration of each factor VIII preparation was measured by a one stage clotting assay and by ELISA, respectively, as described in Materials and Methods, and specific activity was calculated.

[a]Unit/μg

[b]Relative activity (% of wild type)

Table II. Metal Ion Binding Parameters for Factor VIII Wild Type and Mutants

	Ca^{2+} Binding Parameters		Mn^{2+} Binding Parameters		
	K_d (μM)	k	K_d (μM)	k	C^c(% of wild type)
Wild Type	1.18 ± 0.32	15.03 ± 1.09[a] (100.0[b])	1.40 ± 0.24	12.37 ± 0.46[a] (100.0[b])	20.08 ± 0.47[a] (100.0[b])
E110A	n.d.	0.00 ± 0.00 (0.0)	0.09 ± 0.03	1.23 ± 0.13 (10.0)	0.64 ± 0.25 (3.2)
E110D	27.79 ±12.88	1.29 ± 0.13 (8.6)	0.48 ± 0.14	1.95 ± 0.15 (15.8)	1.45 ± 0.16 (7.2)
E113A	0.71 ± 0.24	17.43 ± 1.77 (116.0)	0.39 ± 0.09	15.97 ± 0.99 (129.2)	24.55 ± 1.12 (122.3)
D115A	8.44 ± 1.32	13.72 ± 0.51 (91.3)	0.61 ± 0.25	11.88 ± 1.32 (96.0)	11.56 ± 1.70 (57.5)
D116A	40.38 ± 7.46	6.78 ± 0.27 (45.1)	11.15 ± 2.25	3.33 ± 0.17 (26.9)	3.87 ± 0.23 (19.3)
E122A	37.43 ± 4.00	9.60 ± 0.22 (63.9)	4.11 ± 0.89	6.50 ± 0.33 (52.5)	3.46 ± 0.23 (17.2)
E122D	3.80 ± 1.04	7.06 ± 0.46 (47.0)	1.57 ± 0.37	6.65 ± 0.35 (53.8)	4.76 ± 0.32 (23.7)
E124A	9.51 ± 1.48	9.60 ± 0.35 (63.9)	0.32 ± 0.12	8.71 ± 0.96 (70.5)	6.50 ± 2.07 (32.4)
D125A	n.d.	0.00 ± 0.00 (0.0)	n.d.	0.00 ± 0.00 (0.0)	8.21 ± 0.35 (40.9)
D126A	97.62 ± 18.79	2.18 ± 0.09 (14.5)	0.29 ± 0.11	6.70 ± 0.75 (54.1)	6.17 ± 0.99 (30.7)

Parameter values (k, C, and K_d) were calculated by non-linear least-square regression from the data shown in Figures 3 and 4 using the formula sh in Materials and Methods. n.d.; not determined.

[a]Factor VIII activity is expressed as factor Xa generated (nM/min/nM factor VIII).

[b]Relative activity (% of wild type)

[c]C is the average of the estimated values obtained from Figures 3 and 4.

Fig. 1

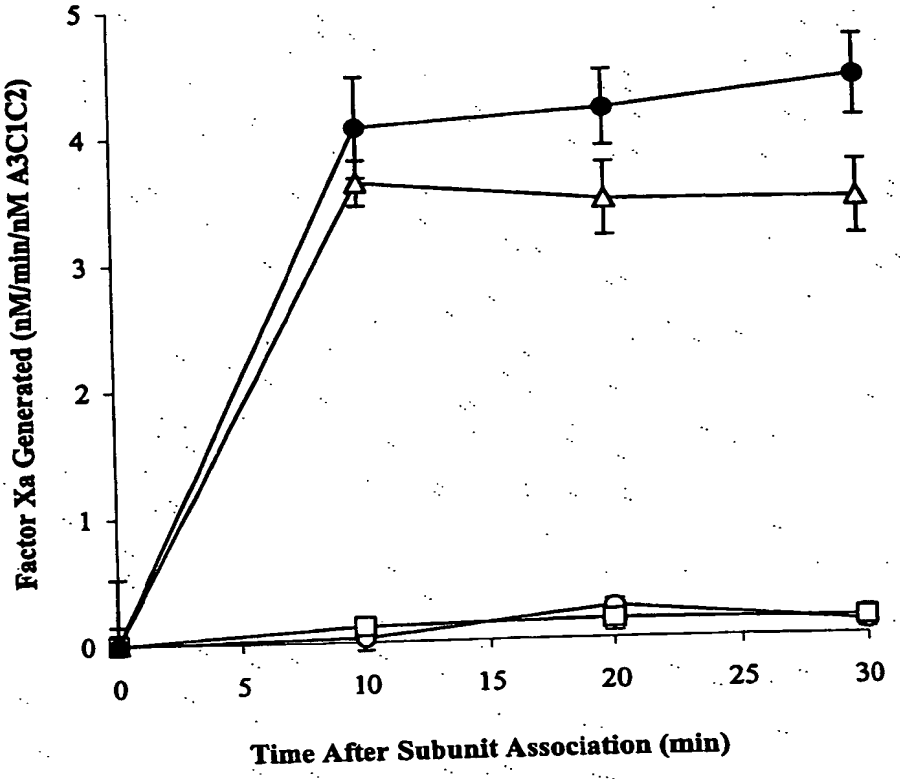
Factor Xa Generated (nM/min/nM A3C1C2)
0
1
2
3
4
5
0
5
10
15
20
25
30
Time After Subunit Association (min)

Fig. 2

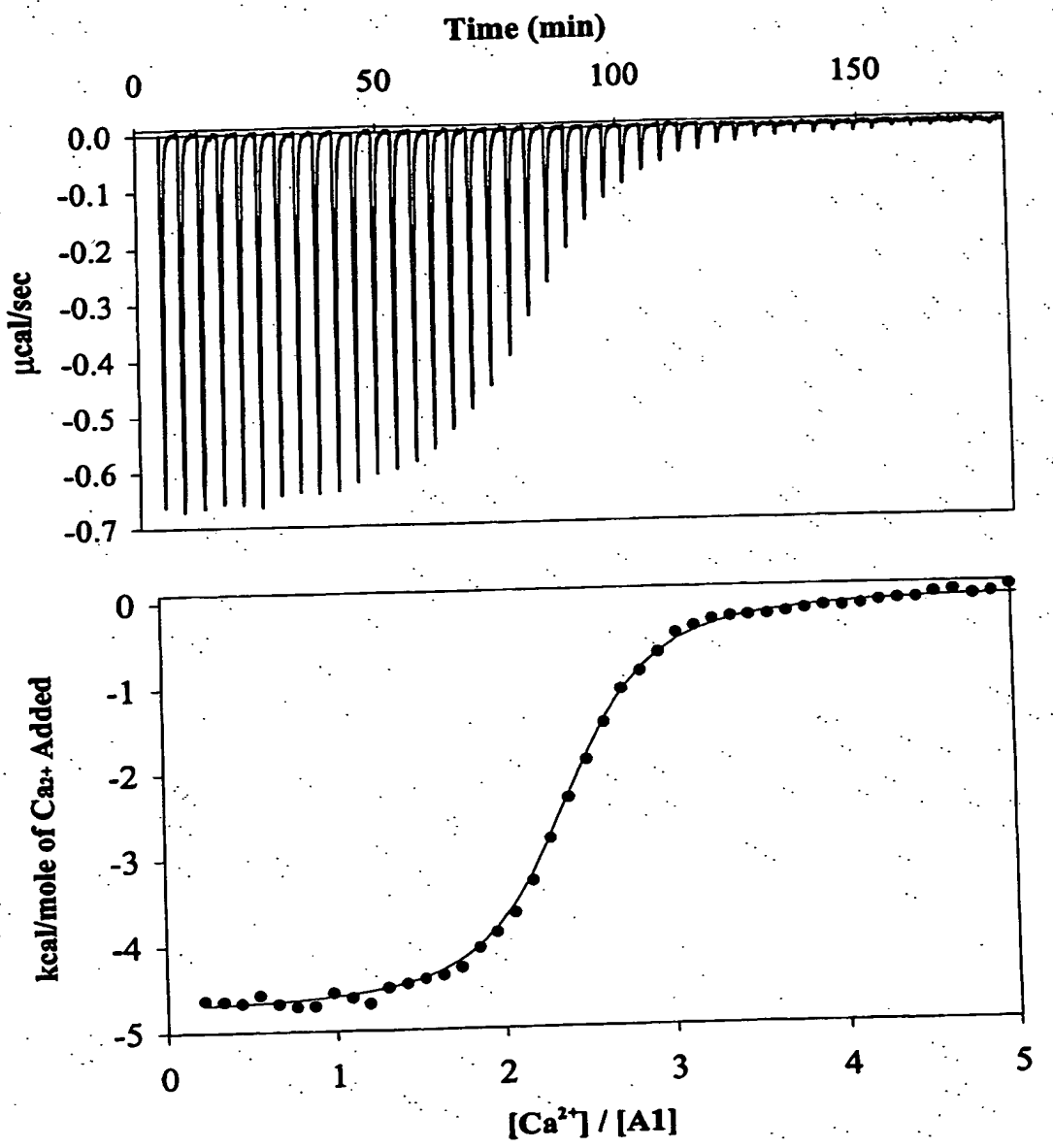
Time (min)
0
50
100
150
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
µcal/sec
0
-1
-2
-3
-4
-5
kcal/mole of Ca2+ Added
0
1
2
3
4
5
[Ca2+] / [A1]

Fig. 3A

A.

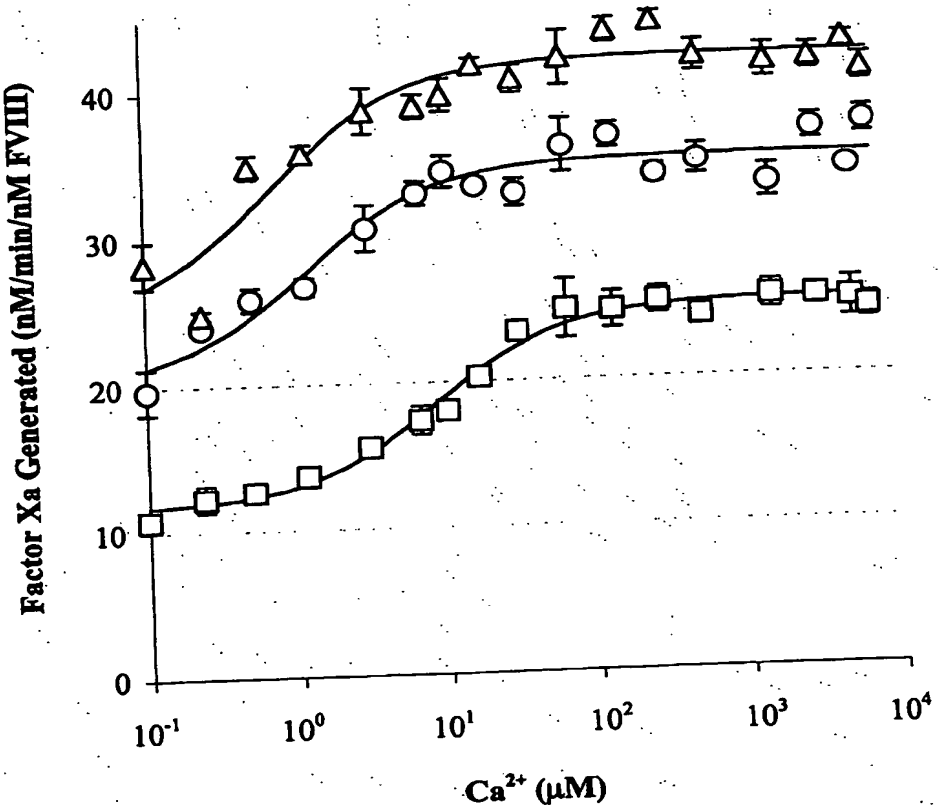
Factor Xa Generated (nM/min/nM FVIII)
40
30
20
10
0
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
Ca^{2+} (μM)

Fig. 3B

B.

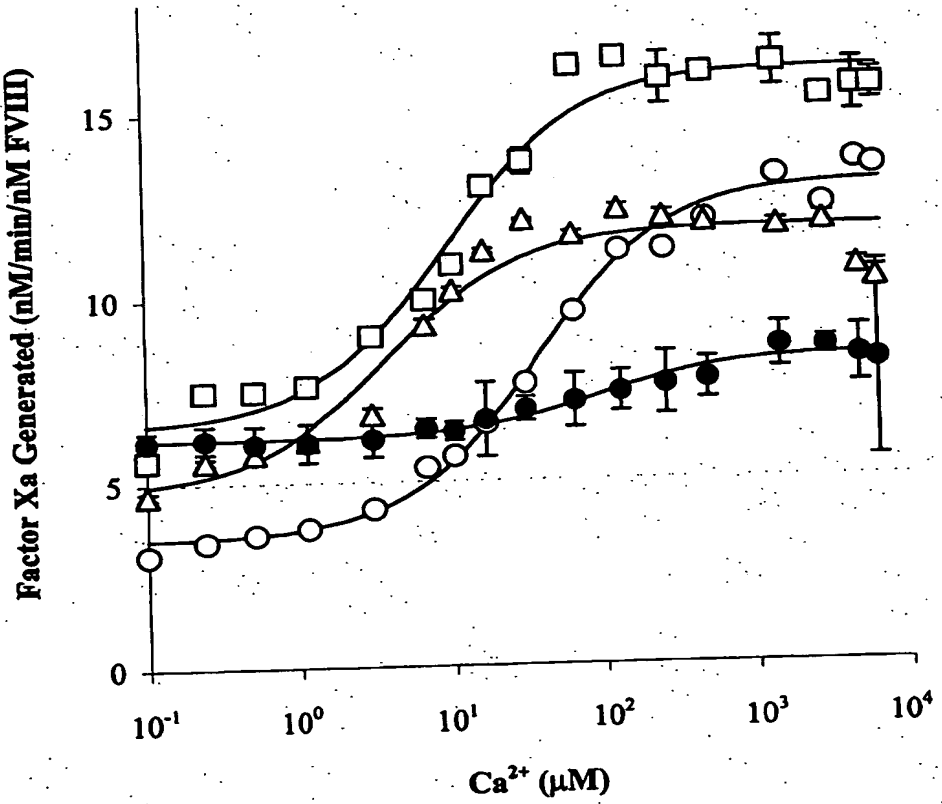
Factor Xa Generated (nM/min/nM FVIII)
15
10
5
0
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
Ca^{2+} (μM)

Fig. 3C

C.

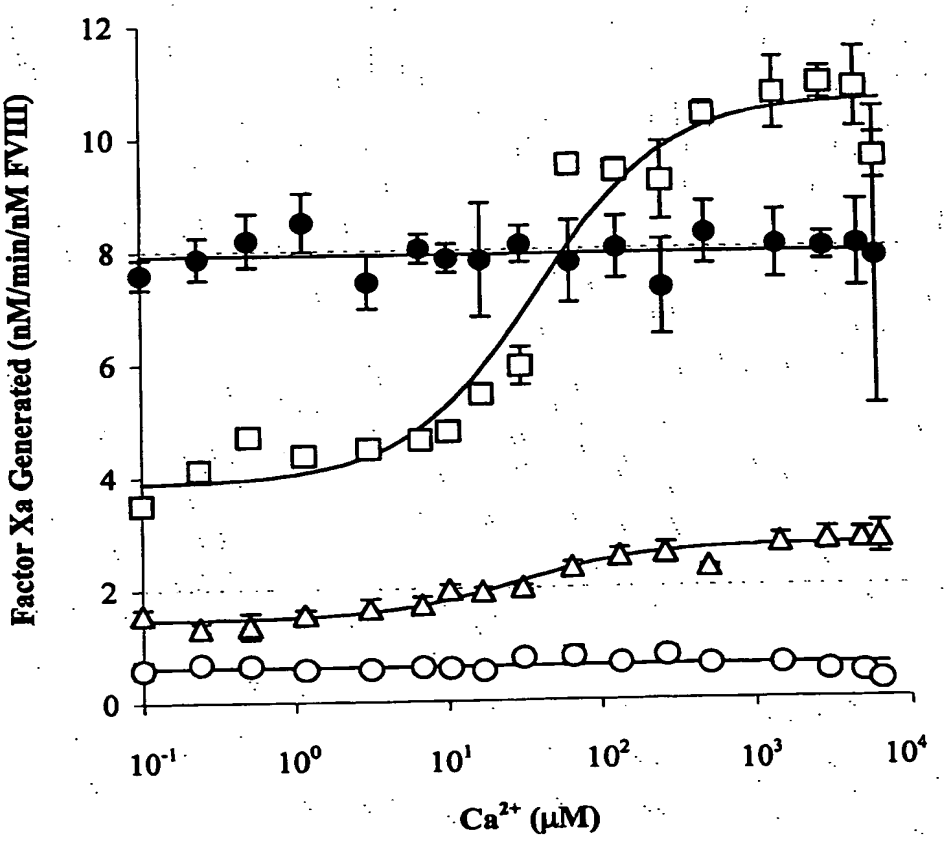
Factor Xa Generated (nM/min/nM FVIII)
12
10
8
6
4
2
0
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
Ca^{2+} (μM)

Fig. 4A

A.

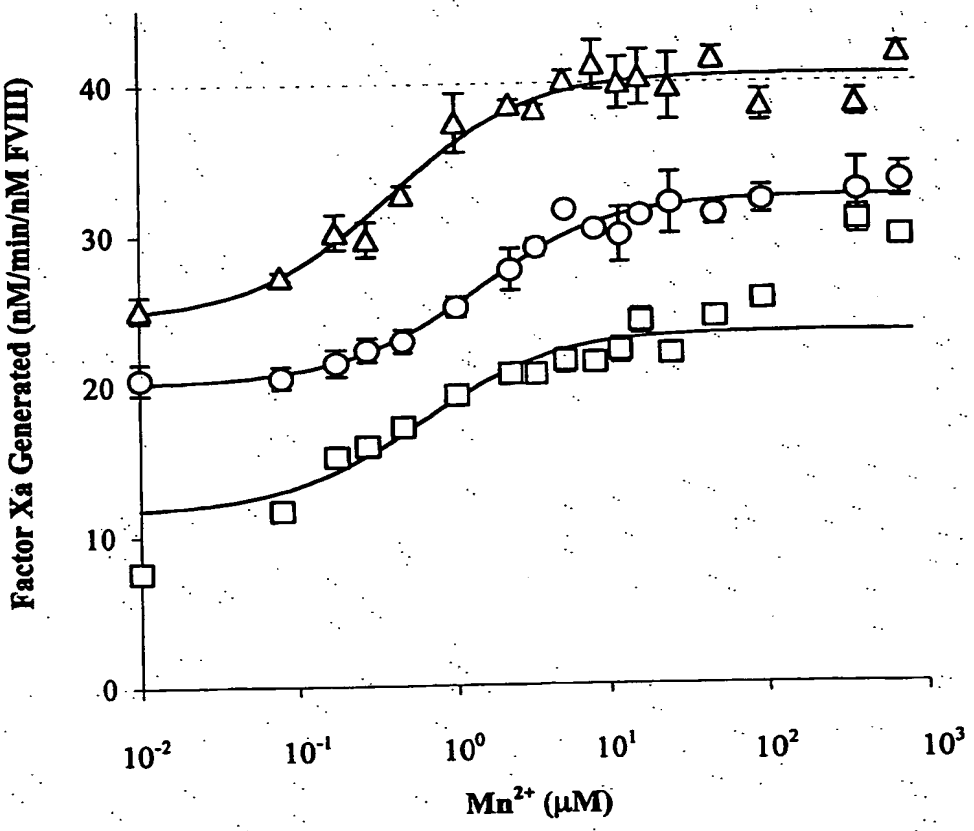
Factor Xa Generated (nM/min/nM FVIII)
40
30
20
10
0
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
Mn^{2+} (μM)

Fig. 4B

B.

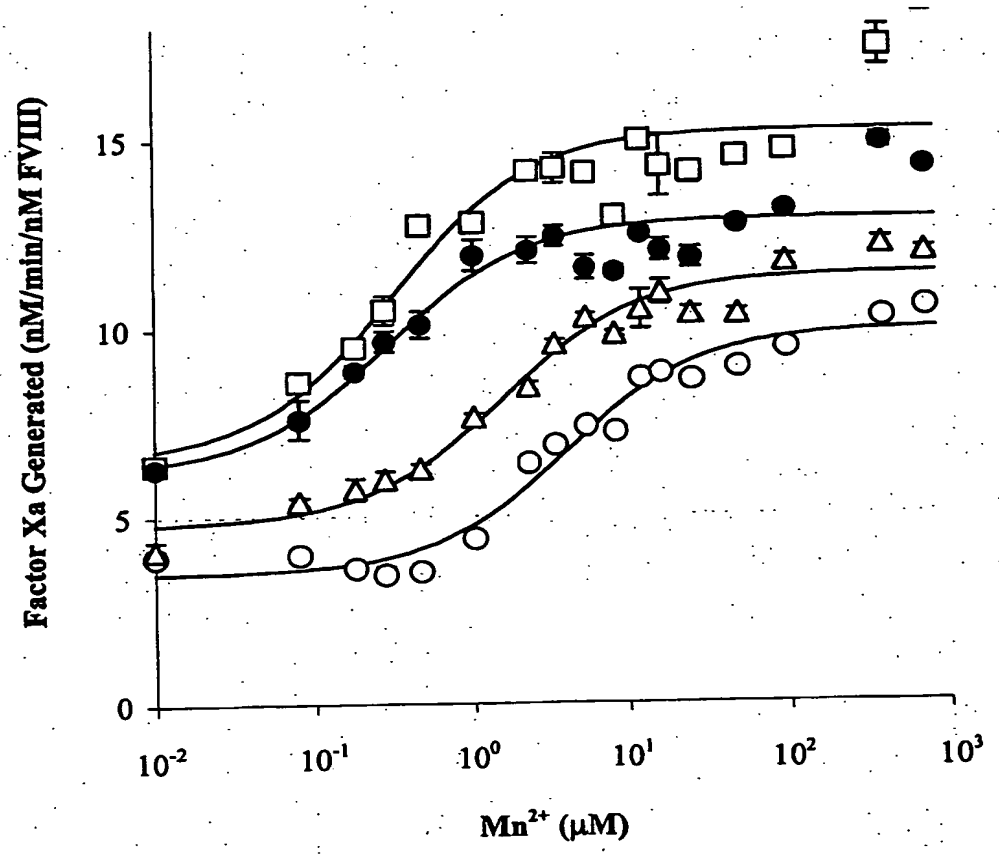
Factor Xa Generated (nM/min/nM FVIII)
15
10
5
0
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
Mn^{2+} (μM)

Fig. 4C

C.

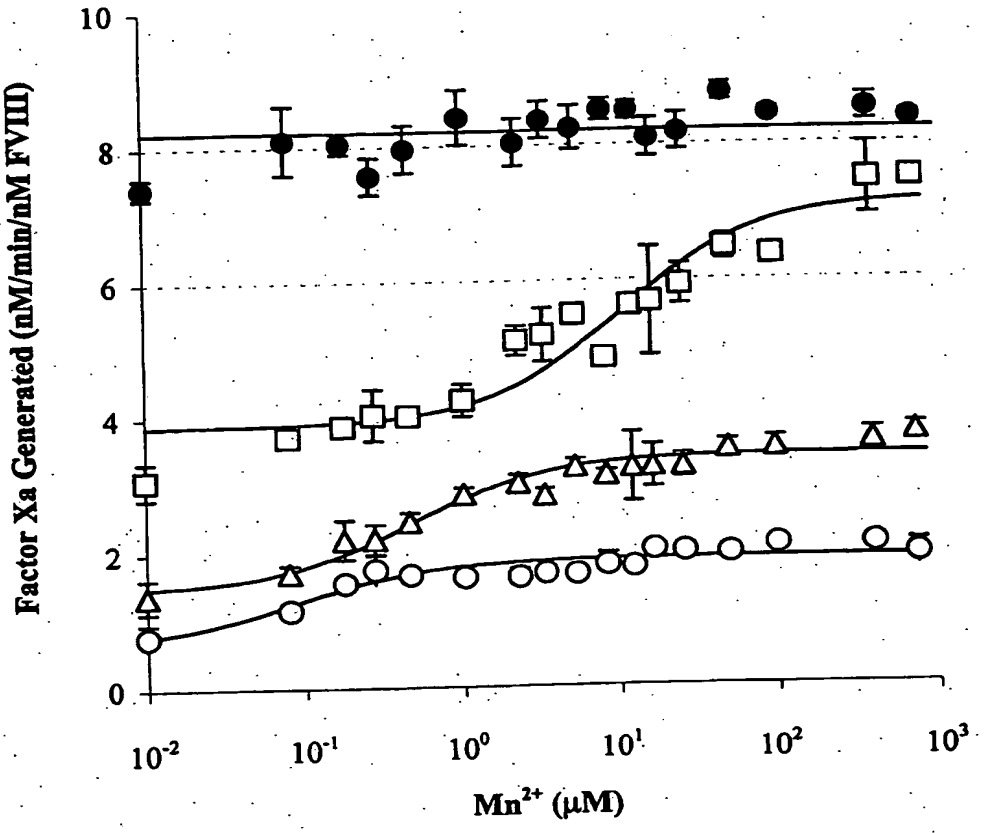
10
8
6
4
2
0
Factor Xa Generated (nM/min/nM FVIII)
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
Mn^{2+} (μM)

Fig. 5

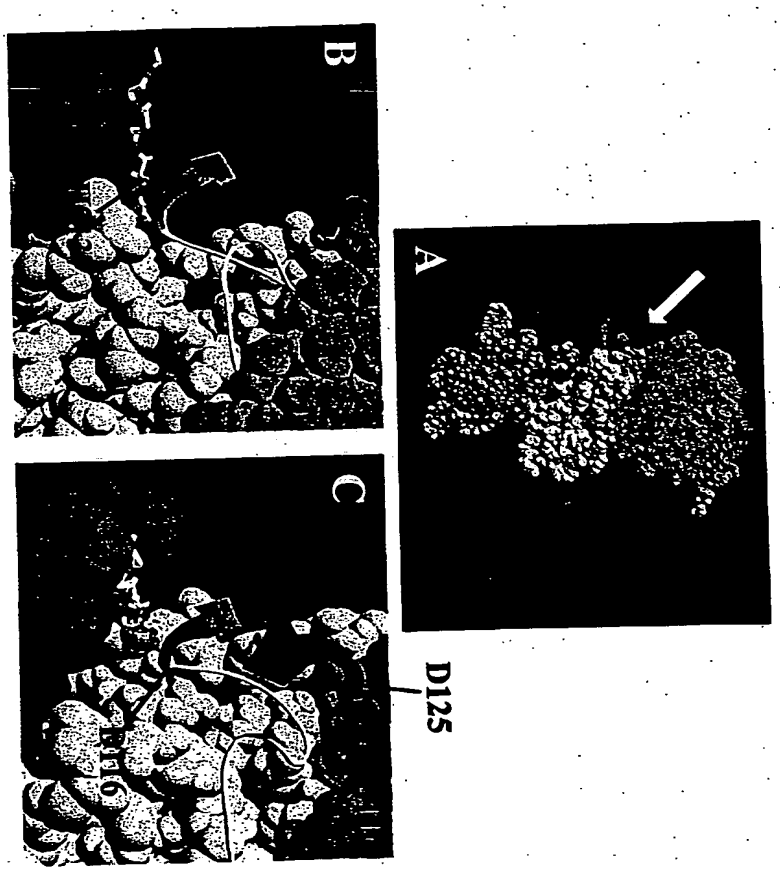

Fig. 6

Factor VIII ^{110}**E**GAEYDD**D**QTSQR**E**KEDD

Factor V ^{96}**E**GASYL**D**HTFPA**E**KMDD

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

WHAT IS CLAIMED:

1. A recombinant factor VIII comprising a point mutation in or near at least one calcium binding site of a wild-type factor VIII, wherein the recombinant factor VIII has a specific activity that is higher than that of the wild-type factor VIII.

2. The recombinant factor VIII according to claim 1, wherein the at least one calcium binding site is in the A1 domain.

3. The recombinant factor VIII according to claim 2, wherein the point mutation comprises a substitution of the amino acid residue at position 113 of SEQ ID NO:2.

4. The recombinant factor VIII according to claim 3, wherein the substitution at residue 113 of SEQ ID NO:2 is selected from the group consisting of E113A, E113V, E113I, E113N, E113L, E113G, and E113M.

5. The recombinant factor VIII according to claim 3, wherein the substitution at residue 113 of SEQ ID NO:2 is E113A.

6. The recombinant factor VIII according to claim 1, wherein the recombinant factor VIII has a specific activity at least about twice as great as the activity of the wild-type factor VIII.

7. The recombinant factor VIII according to claim 1, wherein the recombinant factor VIII has a higher binding affinity for Ca^{2+} compared to that of the wild-type factor VIII.

8. The recombinant factor VIII according to claim 1, wherein the recombinant factor VIII has a higher binding affinity for Mn^{2+} compared to that of the wild-type factor VIII.

9. The recombinant factor VIII according to claim 1, wherein the recombinant factor VIII consists of domains A1, A2, A3, C1, and C2, or portions thereof.

10. The recombinant factor VIII according to claim 9 wherein domains A1 and A2 are present on a heavy chain and domains A3, C1, and C2 are present on a light chain.

11. The recombinant factor VIII according to claim 1, wherein the recombinant factor VIII comprises one or more domains, or portions thereof, from human factor VIII and one or more domains, or portions thereof, from a non-human mammalian factor VIII.

12. The recombinant factor VIII according to claim 1, wherein the recombinant factor VIII has a circulating half-life value that is equivalent to or greater than that of the wild-type factor VIII.

13. The recombinant factor VIII according to claim 1, wherein the recombinant factor VIII is substantially pure.

14. An isolated nucleic acid molecule encoding a recombinant factor VIII according to claim 1.

15. The isolated nucleic acid molecule according to claim 14, wherein the nucleic acid encodes a substitution of the amino acid at position 113 of SEQ ID NO:2.

16. The isolated nucleic acid molecule according to claim 15, wherein the substitution at residue 113 of SEQ ID NO:2 is selected from the group consisting of E113A, E113V, E113I, E113N, E113L, E113G, and E113M.

17. The isolated nucleic acid molecule according to claim 15, wherein the substitution at residue 113 of SEQ ID NO:2 is E113A.

18. The isolated nucleic acid molecule according to claim 14, wherein the nucleic acid is RNA.

19. The isolated nucleic acid molecule according to claim 14, wherein the nucleic acid is DNA.

20. The isolated nucleic acid molecule according to claim 19, wherein the isolated nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO: 1, as modified at codon 113 (nt 337-339).

21. A recombinant DNA expression system comprising a DNA molecule according to claim 19.

22. The recombinant DNA expression system according to claim 21, wherein the DNA molecule is in sense orientation relative to a promoter.

23. A host cell comprising a nucleic acid molecule according to claim 14.

24. A host cell comprising the DNA molecule according to claim 19.

25. The host cell according to claim 24, wherein the DNA molecule is in an expression system.

26. The host cell according to claim 24, wherein the host cell is an animal cell, a bacterial cell, an insect cell, a fungal cell, a yeast cell, a plant cell, or an algal cell.

27. A method of making a recombinant factor VIII comprising:

growing a host cell according to claim 22 under conditions whereby the host cell expresses the ~~recombinant~~ factor VIII; and

isolating the recombinant factor VIII.

28. The method according to claim 27, wherein said growing is carried out *in vitro* in a growth medium.

29. The method according to claim 28, wherein the growth medium comprises von Willebrand Factor.

30. The method according to claim 27, wherein the host cell comprises a transgene encoding von Willebrand Factor.

31. The method according to claim 28, wherein the recombinant factor VIII is secreted into the growth medium, said isolating comprising isolating the recombinant factor VIII from the growth medium.

32. The method according to claim 27 further comprising:

disrupting the host cell prior to said isolating, wherein said isolating comprises isolating the recombinant factor VIII from cellular debris.

33. A method of making a recombinant factor VIII having increased specific activity compared to that of a wild-type factor VIII, the method comprising:

altering the amino acid sequence of wild-type factor VIII to yield a recombinant factor VIII, wherein said altering comprises introducing at least one point mutation in or near at least one calcium binding site of the wild-type factor VIII; and

determining whether the recombinant factor VIII has increased specific activity compared to that of the wild-type factor VIII.

34. A method of treating an animal for hemophilia A, the method comprising:

administering to an animal exhibiting hemophilia A an effective amount of the recombinant factor VIII according to claim 1, whereby the animal exhibits effective blood clotting following vascular injury.

35. The method according to claim 34, wherein the effective amount comprises between about 10 to about 50 units/kg body weight of the animal.

36. The method according to claim 34 wherein the animal is a mammal.

37. The method according to claim 36 wherein the mammal is selected from the group consisting of human, rat, mouse, guinea pig, dog, cat, monkey, chimpanzee, orangutan, cow, horse, sheep, pig, goat, rabbit, and chicken.

38. The method according to claim 34 further comprising:
periodically repeating said administering.

39. A pharmaceutical composition comprising the recombinant factor VIII according to claim 1.

40. The pharmaceutical composition according to claim 39 further comprising a stabilizer.

41. The pharmaceutical composition according to claim 39 further comprising a delivery vehicle.

42. The pharmaceutical composition according to claim 39 further comprising a carrier.

43. The recombinant factor VIII according to claim 1 wherein the recombinant factor VIII further comprises modified inactivation cleavage sites.

44. The recombinant factor VIII according to claim 1 wherein the recombinant factor VIII further comprises factor IXa and/or factor X binding domains modified to enhance the affinity of the recombinant factor VIII for one or both of factor IXa and factor X.

45. The recombinant factor VIII according to claim 1 wherein the recombinant factor VIII further comprises modified sites that enhance secretion in culture.

46. The recombinant factor VIII according to claim 1 wherein the recombinant factor VIII further comprises modified serum protein binding sites that enhance the circulating half-life thereof.

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